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Research Centre in Physical Activity, Health and Leisure (CIAFEL)

SEDENTARY BEHAVIOUR IMPAIRS THE SKELETAL MUSCLE REPAIR INDUCED BY TOXIC INJURY IN AN ANIMAL MODEL

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KEYWORDS: REGENERATION; FIBROSIS; SATELLITE CELLS; LOADING; PHYSICAL INACTIVITY

I'm glad to be here right now, poking at my threshold.
I want to get more comfortable being uncomfortable.
I want to get more confident being uncertain.
I don't want to shrink back just because something isn't easy.
I want to push back, and make more room in the area between I can't and I can.
Maybe that spot is called I will!

Kristin Armstrong

To my daughter Mia, and my loving family

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EXPERIMENTAL STUDIES

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Resumo

Estudos recentes demonstram que o sedentarismo é um fator de risco independente que afeta negativamente o estado de saúde geral. Os seus diversos efeitos promovem disfuncionalidade em vários órgãos e sistemas. Dos diferentes órgãos afetados, o músculo esquelético – frequentemente submetido a estímulos lesivos e caracterizado pela sua grande plasticidade – é extremamente vulnerável a esta condição. Contudo, apesar dos efeitos negativos do sedentarismo na sua morfologia e funcionalidade estarem bem documentados, há pouca evidência que demonstre o seu efeito durante o processo de reparação muscular (RM). Sabendo que este processo é decisivo para a determinação do fenótipo do músculo esquelético ao longo da vida, determinando tanto a sua qualidade como a sua funcionalidade máxima, parece ser pertinente estudar os efeitos do sedentarismo na RM. Assim, este trabalho verificou se o sedentarismo tem efeitos nocivos na RM (estudo 1), e na resposta inflamatória local (estudo 2) em ratos *Wistar* submetidos a lesão muscular induzida por cardiotoxina (CTX). Para tal, os ratos, alojados individualmente, passaram 8 semanas em gaiolas sem acesso a roda livre, o grupo sedentário (SED), ou em gaiolas com acesso a roda livre e com a possibilidade de correr voluntariamente, o grupo controlo (EX). Posteriormente, todos os ratos foram injetados com CTX no tibial anterior direito (músculos CTX) e com soro no tibial anterior esquerdo (músculos Sham), e foram sacrificados nos dias seguintes à injeção. Foram realizadas análises histológicas e imunohistoquímicas dentro da área lesada para a quantificação da percentagem de miotúbulos e determinação da sua fase de desenvolvimento, para a quantificação de fibrose, e para a determinação do número de neutrófilos e macrófagos, do tipo 1 e 2. Os resultados obtidos demonstram que o sedentarismo deteriora a RM, após lesão induzida por CTX, favorecendo o aumentado da deposição de fibrose, em detrimento do crescimento e maturação miotubular. Este efeito geral parece ser suportado pelo facto do sedentarismo: alterar a resposta inflamatória local (intensificando o estado pró-inflamatório e inibindo o estado anti-inflamatório); diminuir a funcionalidade dos miotúbulos (tanto a sua taxa de formação como o seu processo de maturação e crescimento); e alterando a funcionalidade dos

fibroblastos (promovendo a formação de fibrose). Assim, tendo em conta a importância dos músculos esqueléticos para a manutenção da qualidade de vida e para a potencialidade na diminuição do risco de doenças, os nossos resultados demonstram que o sedentarismo promove efeitos extremamente nocivos durante a RM, favorecendo a fibrose. Consequentemente, estes efeitos irão aumentar a possibilidade dos músculos esqueléticos serem, progressivamente, substituídos por tecido conjuntivo, resultando numa progressiva perda de qualidade e máxima funcionalidade ao longo da vida.

PALAVRAS CHAVE: REGENERAÇÃO; FIBROSE; CÉLULAS SATÉLITE; CARGA; INATIVIDADE FÍSICA

Abstract

Recent evidence demonstrates that sedentary behaviour (SB) negatively impacts health independent of other factors. Indeed, the SB-related deleterious effects effectively promote multiple organ dysfunction. From the several organs disturbed, the skeletal muscles – often subjected to various stressful conditions, and characterised by great plasticity – are highly susceptible to SB. However, despite the increasing data addressing SB-related effects on muscle functionality and morphology, less is known regarding its effects on skeletal muscle repair (SMR) process. Knowing that the SMR outcome is pivotal in governing the skeletal muscles phenotype during the life time, determining its quality, and, therefore its maximal functionality, it seems relevant to study the SB effects during the SMR process. Thus, this work addressed if SB has detrimental effects on SMR (study 1) and if it modulates the tissue inflammatory response (study 2) in male *Wistar* rats after cardiotoxin (CTX)-induced injury. Individually caged rats spent 8 weeks either as a sedentary group (SED) – in cages without running wheel – or as a control group (EX) – in cages with running wheel for voluntary running. Subsequently, all rats had each tibial anterior muscles infused either with CTX (CTX; right muscle) or saline solution (Sham; left muscle), and were sacrificed on the following days post-injection. Histological and immunohistochemical analyses from the muscle damaged area were used for calculating the myotubes percentage and their developmental phase, the fibrosis accretion, and for measuring the number of neutrophils, and M1 and M2 macrophages subtypes. Our results show that SB impairs rat SMR after CTX-induced damage, supporting fibrosis instead of myotubes growth and maturation. This overall outcome seems to be supported by the negative effects of SB on the local inflammatory response (exacerbating the Th1 phase, and impairing the Th2 phase); on the myotubes functionality (postponing their formation rate, maturation and growth); and on the fibroblasts activity (increasing the fibrotic tissue accretion). Our results clearly show that SB greatly prejudices the SMR process, favouring scar tissue formation. Consequently, considering the importance of skeletal muscles on the overall quality of life and ability to decrease the

predisposition to diseases, these SB-related effects will increase the skeletal muscles possibility to be progressively substituted by connective tissue, when coping with daily wear and tear, resulting in loss of their quality and maximal functionality throughout the lifespan.

KEYWORDS: REGENERATION; FIBROSIS; SATELLITE CELLS; LOADING; PHYSICAL INACTIVITY

List of Abbreviations

CD: cluster differentiation

CSA: cross-sectional area

CTX: cardiotoxin

dpi: day post-injury

ECM: extra cellular matrix

EX: exercise control group

FAPs: fibro\adipogenic progenitors

FT: fibrotic tissue

H&E: haematoxylin and eosin

IQR: interquartile range

IL: interleukin

ip: intraperitoneally

MET: metabolic equivalent

MMPs: matrix metalloproteinases

SB: sedentary behaviour

SCs: satellite cells

SD: standard deviation

SED: sedentary group

SMR: skeletal muscle repair

SMReg: skeletal muscle regeneration

SR: picrosirius red

1. GENERAL INTRODUCTION

Sedentary behaviour overall effects

Lack of voluntary skeletal muscle activity, also known as sedentary behaviour (SB) – any waking behaviour inducing an energy expenditure ≤1.5 metabolic equivalent (MET) during sitting or reclined position (Sedentary Behaviour Research, 2012) – is a rapidly growing field of research. Recent evidence linking SB with all-cause mortality, cardiovascular disease, type 2 diabetes and metabolic syndrome, and some types of cancer has been thoroughly addressed (de Rezende et al., 2014; Thyfault et al., 2015). In fact, SB not only substantially decreases the total years of life and their quality, but also deeply impairs multiple organs functionality, inducing several and general tissue maladaptation (Booth et al., 2012). Besides these overall detrimental effects, increasing data is also showing the SB-related effects on skeletal muscle functionality. For example, recent studies demonstrate that SB impairs the skeletal muscle metabolic capacity, i.e., prejudicing glucose metabolism and fat oxidation, and promoting mitochondrial dysfunction (Rynders et al., 2017). Indeed, SB severely affects the skeletal muscle tissue phenotype, its morphology and function, e.g., endorsing skeletal muscle atrophy and loss of strength (Booth et al., 2012; Fiuza-Luces et al., 2013). Moreover, some studies evince the relationship between the skeletal muscle mass and the survival prediction capacity in older adults (Srikanthan & Karlamangla, 2014), and the relationship of muscle function, i.e., muscle strength, with premature death in male adolescents (Ortega et al., 2012). These considerable amounts of data corroborate the increasingly importance of maintaining a healthy and functional skeletal muscle.

Regular exercise and physical activity effects

Interestingly, paralleled to this growing field of research, several groups are comprehensively investigating the so-called “exercise is medicine”, i.e., the general positive effects of regular exercise and/or physical activity on the prevention and treatment of several chronic diseases (Lobelo et al., 2014). This rapidly increasing research area is supported by (1) epidemiological evidence

showing that regular exercise has many therapeutic and preventive effects on various diseases – curiously some research groups advocate that exercise functions like a polypill (Fiuza-Luces et al., 2013); (2) several recent studies demonstrating that, when appropriately recruited, the skeletal muscle tissue is able to produce several cytokines (commonly known as myokines) that effectively produce positive systemic effects in other organs and tissues, by endocrine signalling, and locally, by paracrine or autocrine signalling (Pedersen, 2009, 2013; Whitham & Febbraio, 2016); and (3) data showing that both acute and chronic regular exercise effectively modulate systemic inflammation, and has a plausible role in exerting significant anti-inflammatory effects (Gleeson et al., 2011). Thus, it is clear that the literature has strong evidences demonstrating both the detrimental effects of SB on the multiple organs functionality and, specifically, on the skeletal muscle phenotype, and the beneficial effects of regular exercise. Nonetheless, less is known regarding the possible effects of different levels of physical activity on the important physiological mechanism that regulates the skeletal muscle phenotype during the lifespan, i.e., the skeletal muscle repair.

The skeletal muscle repair process

The skeletal muscle repair (SMR) is a very intricate process, comprising a coordinated interaction of multiple cells, that may determine the development of a favourable skeletal muscle phenotype – when the damaged fibres are replaced by new ones, and not by fat or fibrosis, i.e., skeletal muscle regeneration (SMReg) – with consequent morphological and functional maintenance; or an adverse phenotype – upon substitution of the damaged fibres by fat or scar tissue – with subsequent morphological and functional impairment (Moyer & Wagner, 2011). Briefly, SMR comprises two distinct phases: firstly, upon injury, the pro-inflammatory immune system response – usually referred as Th1 phase, and mainly accomplished by pro-inflammatory neutrophils and M1 macrophages – promotes cellular debris and necrotic tissues clearance, and, concurrently, the activation and proliferation of satellite cells (SCs) and/or myogenic stem cells, fibroblasts, (Tidball, 2017; Tidball & Villalta, 2010) and fibro\adipogenic

progenitors (FAPs) (Joe et al., 2010). This phase culminates with an increased number of proliferating myogenic progenitor cells (Tidball, 2017; Tidball & Villalta, 2010) and fibroblasts that start an atypical deposition of fibrotic tissue in the extra cellular matrix (ECM) (Moyer & Wagner, 2011). Secondly, following this proliferative/cleaning period, the anti-inflammatory immune system response, the Th2 phase, mainly accomplished by anti-inflammatory M2 macrophages, supports myoblasts fusion and terminal differentiation, and tissue resolution (Tidball, 2017; Tidball & Villalta, 2010). This occurrence is paralleled with a reduction in fibroblasts and FAPs number, alongside with an ECM remodelling, operated mainly by matrix metalloproteinases (MMPs) (Moyer & Wagner, 2011), i.e., proteases that degrade ECM (mainly collagen), in order to reduce the excessive amount of fibrotic tissue initially synthesized. Regardless of the initial fibrosis and its scaffold importance, setting a structural support for the SMReg, the SMR may be compromised when there is an excessive fibrotic production during the Th1 phase, and/or when, during the Th2 phase, the later ECM remodelling fails on removing the unnecessary added fibrotic tissue, circumstance that will consequently develop a more fibrotic and dysfunctional muscle phenotype. Considering the presented data, the SMR balances between SMReg and fibrosis, being more successful, as already mentioned, if it develops a more functional phenotype, i.e., if it ends on adequate myofibers replacement rather than their substitution by fibrotic or fat tissue. Nevertheless, the plausible detrimental effects of prolonged SB in the skeletal muscle myogenic potency, and its modulating effects in the SMR process, shown to be pivotal in regulating the skeletal muscle phenotype, remain poorly addressed. Indeed, considering that skeletal muscles are organs frequently subjected to various stressful conditions, like the mechanical, metabolic, thermic and oxidative stress, the consequent damage to muscle fibres substantiate the day-to-day wear and tear. Consequently, if the repeated insults are effectively repaired by SMReg, the muscles tend to maintain their quality and function, otherwise, if the muscles fibres are recurrently substituted by scar tissue, the muscles progressively lose their maximal functionality, which compromises the ability to restore systemic homeostasis imbalances increasing predisposition to diseases, with a decreased

capacity to perform muscular work and overall impaired quality of life (Booth et al., 2012).

It is known that several factors like age, genetics, type of insult and its severity may influence SMR outcome, and, intriguingly, it is also known that skeletal muscle loading may also modulate this capacity. Indeed, augmented muscle recruitment, through regular exercise and enhanced physical activity, seems to be beneficial in both improving SCs functionality, their development and maturation processes, i.e., increasing myogenic potency, and impairing fibrosis during SMR (Teixeira & Duarte, 2016). Moreover, several studies suggest that different patterns of muscle recruitment may interact with the immune system cellular response. Indeed, some studies showed that both acute exercise (60 min of swimming) seems to effectively increase the circulating monocytes/macrophages phagocytic capacity (Silveira et al., 2007), and also chronic exercise (voluntary running) positively modulated both macrophages and lymphocytes cytokine production (Sugiura et al., 2002). Furthermore, both acute and chronic muscle loading conditions, also seem to increase macrophages tumor cytotoxicity and chemotaxis capacities (Woods et al., 2000). Interestingly, the effects of SB or increased voluntary physical activity on the overall SMR cellular dynamics, i.e., SCs, fibroblasts, and immune cells functions, and their interaction, remain undetermined.

Hypotheses and general organization

Considering the questions raised, the global purpose of this thesis is to scrutinise how prolonged SB in *Wistar* rats, achieved through restricted physical activity, affects the overall quality of the SMR process after a chemical insult – studying the skeletal muscle inflammatory response, the subsequent SCs activity and the fibrotic tissue accretion. The working hypotheses to be tested are:

1. The SB negatively affects the SMR, impairing SCs activity and favouring scar tissue accretion.
2. The SB influences the Th1 inflammatory phase, comprising the tissue resolution, and impairs the subsequent Th2 phase.

The present work is organized into five chapters: Chapter 1 provides a broad general introduction which addresses the overall theoretical background and supports the rationale for this work; Chapter 2 is constituted by a narrative review article, mainly exploring the skeletal muscle loading potential effects on its regenerative capacity; Chapter 3 is comprised by two experimental studies. The first study addresses the plausible effects of SB during the SMR after a chemical injury, mainly analysing the histomorphological features of the healing process; the second study analyses the SB-related effects on the skeletal muscle inflammatory response and its consequences on the overall SMR; Chapter 4 comprises the overall discussion; and, finally, in Chapter 5 the main conclusions of the present work and perspectives for future research are presented.

2. NARRATIVE REVIEW ARTICLE

Skeletal Muscle Loading Changes its Regenerative Capacity

Eduardo Teixeira & José Alberto Duarte

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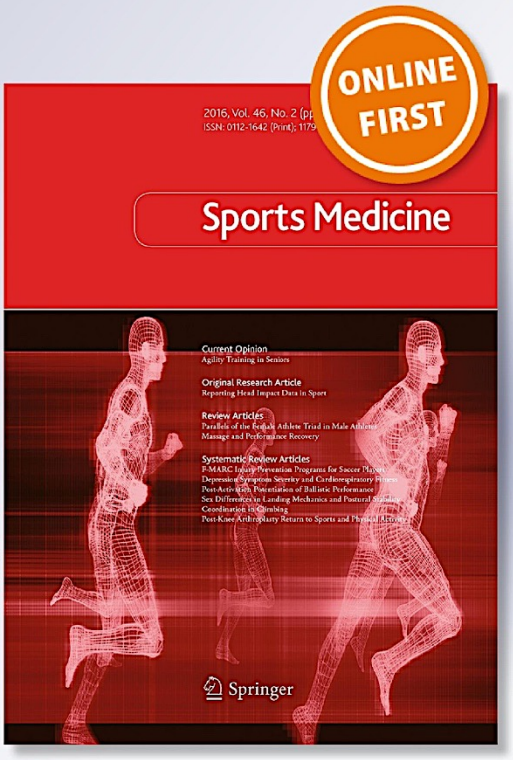

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[Review Article]

Skeletal Muscle Loading Changes its Regenerative Capacity

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REVIEW ARTICLE

Skeletal Muscle Loading Changes its Regenerative Capacity

Eduardo Teixeira[1] · **José Alberto Duarte**[1]

Abstract Whenever skeletal muscle insults occur, both by functional impositions or other injury forms, skeletal muscle repair (SMR) follows. The SMR succeeds when proper skeletal muscle regeneration and limited fibrosis ensue. Muscle fiber replenishment by fibrosis negatively affects the tissue quality and functionality and, furthermore, represents the worst post-injury phenotypic adaptation. Acute muscle injury treatment commonly follows the RICE method—rest, ice, compression, and elevation. This immediate immobilization seems to be beneficial to preserving the tissue structure and avoiding further destruction; however, if these interventions are delayed, the risk of muscle atrophy and its deleterious-related effects increase, with resultant impaired SMR. Moreover, a growing body of evidence shows positive skeletal muscle loading (SML) effects during SMR since it seems to effectively increase satellite cells (SCs) in their activation, proliferation, self-renewal, and differentiation capacities. Additionally, recent data show that SML may also influence the functions of other participants in SMR, compelling SMR to achieve less fibrotic accretion and accelerated muscle mass recovery. Moreover, given the SML effects on SCs, it is plausible to consider that these can increase the myofibers' basal myogenic potential. Thus, it seems relevant to scrutinize the possible acute and chronic SML therapeutic and prophylactic effects regarding the SMR process.

Key Points

Acute immobilization upon injury may be beneficial but can compromise proper skeletal muscle repair (SMR).

Skeletal muscle loading (SML) may increase the number of satellite cells, their proliferation, and their differentiation capacities, which enhance proper SMR.

SML increases vascularization and collagen turnover and should therefore be promoted during SMR.

1 Introduction

The skeletal muscle potency to respond to different stimuli such as exercise, immobilization, trauma, or chemical insult, relies on its regenerative capacity due to the presence of a myogenic stem cell population known as satellite cells (SCs) [1]. The SCs, resting between the sarcolemma and the skeletal muscle basal lamina, first described by Alexander Mauro [2], are the prime source of myogenic cells. Nonetheless, the fact that stem cells from other tissues may effectively migrate, incorporate the SC pool, or acquire its properties and myogenic potential [3–5] reveals that skeletal muscle regeneration (SMReg) may be affected by other stem cells and chemoattractants released by skeletal muscle fibers. SMReg is defined when damaged myofibers, or their segments, are replaced by new ones, without modifying the original tissue structure. However,

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skeletal muscle repair (SMR), depending on the injury's intricate nature, i.e. insult type and severity, does not only occur by SMReg but also by the total or partial substitution of damaged fibers by connective tissue (fat and scar tissue) with loss of original structure and functional impairment [6, 7]. Ideally, in order to maintain skeletal muscle structure and functionality, the SMR should always be achieved by SMReg.

Muscle injuries may range from: (1) mild (focal intracellular damage, i.e. sarcoplasm rupture or myofibril disruption); (2) moderate (myofibril complete or segmental injury or necrosis); and (3) severe (affecting one or more muscle bundles, blood vessels and interstitial tissues). To cope appropriately with these different insults, various participants execute numerous functions composing the SMR per se, which can be characterized by either (1) SMReg through activation, proliferation, and differentiation of SCs into myoblasts, during mild injuries and day-to-day wear and tear; (2) activation of mononucleated cells (fibroblasts, myogenic, and resident inflammatory cells), promoting both SMReg and extracellular matrix (ECM) remodeling (fibrotic accretion or degradation) during moderate injuries or, finally, during severe insults; (3) activation of all the cells mentioned in points (2) and (3) and, through chemotactic signaling released into the blood stream, infiltration of an additional variety of inflammatory and myogenic cells [6, 8–10]. During moderate-to-severe insults, SMR also embraces inflammatory response, characterized by a complex cellular and molecular event that interacts with the activity of SCs throughout the process [10]. Considering these wide interactions, knowledge regarding SMReg cellular and molecular mechanisms is pivotal when analyzing SMR. Nonetheless, recent studies show that both ECM characteristics [11] and their constituents [12] seem to be fundamental for proper SMR and SC behavior, indicating that the ECM is another important participant during SMR. Moreover, since skeletal muscle loading (SML) effectively modulates ECM turnover [13], it seems relevant to analyze its SMR-related interactions.

Curiously, therapeutic approaches such as surgical techniques for scar tissue removal, biologic scaffold grafting, and use of nonsteroidal anti-inflammatory drugs (NSAIDs) and antifibrotic agents [14] have been used to accomplish effective SMR. Additionally, RICE (rest, ice, compression, and elevation) continues to be the most used procedure to acutely treat muscle injuries. Although RICE may effectively reduce the injury bleeding, preventing further tissue damage during the initial post-injury hours [15], if the forced immobilization continues, as it usually does, it may also contribute to an increased skeletal muscle atrophy through muscular unloading, which exacerbates many adverse consequences such as increased proteolysis, through activation of ubiquitin-proteasome, lysosomal, and calpain proteolytic pathways, and increased myonuclei loss through nuclear apoptosis [16], impairing a proper SMReg and, therefore, the SMR.

This raises a pertinent issue for those seeking a faster and less fibrotic SMR, and highlights a plausible SML importance during the process. Indeed, previous studies showed favorable SML effects during SMR [17]. As addressed in Sect. 2.1.1, recent data show that SML seems to efficiently stimulate the activation [18] and proliferation capacities [19] of SCs. These advantageous effects seem to be important during animal growth [20], and seem to be conserved during human aging [19], which putatively ensures an increased basal myogenic potential (BMP). The BMP concept, i.e. the putative SC reserve that will turn into new muscle fibers if necessary, is supported by (1) recent animal studies that, although relatively controversial, indicate SCs as absolutely necessary for SMReg after muscle injury [21, 22]; (2) evidence supporting the fact that the number and functionality of SCs are critical to preventing muscle tissue substitution by fat or fibrosis after muscle damage [7]; and (3) data showing that reduced muscle regeneration, after immobilization-related atrophy in elderly humans, was related to a blunted response in SC proliferation [23].

Consequently, it seems appealing to scrutinize (1) the SML effects on the skeletal muscle BMP, i.e. its ability to acutely and chronically increase the number of SCs; (2) its ability to induce milieu alterations on both SCs and ECM; (3) its capacity to modulate SMR outcomes; and (4) its likely prophylactic role against skeletal muscle insults.

In order to provide the framework for this article's discussion, we define SML as muscle requirements equal to or higher than those used in day-to-day wear and tear, while, conversely, skeletal muscle unloading occurs during prolonged bed rest or limb immobilization, and favors muscle deconditioning and atrophy.

2 Skeletal Muscle Loading (SML) Effects on Skeletal Muscle Stem Cells and Their Milieu

Recent evidence indicates that the ability of stem cells to proliferate, self-renew, or differentiate into a specific phenotype may be controlled by their particular mechanical environment, i.e. stiffness alterations of their milieu, since they are able to perceive and react to external mechanical forces [24–26]. Experiments with cultured mesenchymal stem cells showed that stiffness manipulation of the collagen-coated gels was able to determine their specific lineage and phenotype and, subsequently, that soft gels simulating brain matrices were neurogenic, rigid gels simulating muscle were myogenic, and the denser gels were osteogenic [27]. Moreover, this study also showed the

importance of the chemical milieu of stem cells since manipulation of soluble factors was able to reprogram cell lineage specification in the early phases of differentiation. Therefore, both mechanical and chemical factors seem to be important in governing the function and fate of stem cells. Like other stem cells, SCs are located in a very specific physical environment, comprising the ECM, vascular and neural tissues, different cell types, and numerous diffusible molecules. All these constituents interact with each other in order to precisely regulate the quiescence, self-renewal, proliferation, and differentiation of SCs. Collectively, the immediate niche, local milieu, and systemic milieu may stimulate the activity of SCs. Briefly, the immediate niche comprises the regulatory signaling pathways (Wnt, Notch, and sphingolipid signaling), the myofiber niche (secretion of stromal cell-derived factor-1 [SDF-1], transmembrane Notch ligand Delta), and the ECM and related factors (hepatocyte growth factor [HGF], fibroblast growth factor [FGFs], insulin growth factor [IGF] and matrix metalloproteinases [MMPs]). The local milieu comprises interstitial cells (fibroblasts, myogenic precursors, and fibro/adipogenic precursors), motor neurons (secrete the neurotrophins nerve growth factor [NGF] and brain-derived neurotrophic factor [BDNF]), and vasculature (secrete vascular endothelial growth factor [VEGF]). Finally, the systemic milieu comprises immune cells, interleukin (IL)-6, androgens, and nitric oxide ($^{\cdot}$NO) [4]. The effects of SML on the SCs niche are addressed in Sect. 2.1.3.

Recently, some studies showed that muscle tissue is able to produce, upon SML, several myokines, i.e. cytokines that exert numerous effects by endocrine, paracrine, or autocrine signaling [28–30]. This may indicate that SML, in addition to the loading-derived mechanical factor, also produces chemical signals that may be critical to either the behavior of the SCs or the incorporation of other myogenic stem cells. The effects of SML on the SC pool are addressed in Sect. 2.1.

2.1 SML Effects on Satellite Cells

The skeletal muscle adaptation concept, regarding the functionality of the SCs, continues to embrace different features of skeletal muscle plasticity such as growth, hypertrophy, atrophy, nuclear turnover, aging, and SMReg. Recently, the interaction between the biology of SCs and skeletal muscle adaptation has been the subject of intense debate [31–34]. Human studies examining the functionality of SCs during different features of muscle fiber adaptation (e.g. exercise during aging [35, 36], skeletal muscle responses following resistance [36, 37], endurance training [35], remodeling [38]) are defining new perspectives for the study of SC functions during muscle plasticity. This section will highlight the current data regarding the response of SCs to SML.

2.1.1 Acute Effects

A very precise myogenic program, coordinated by key transcription factors, the myogenic regulatory factors (MRFs), controls the quiescence, activation, proliferation, and differentiation or self-renewal activities of SCs. First, SCs are mitotically quiescent (G_0 phase of cell cycle) and express Pax7 but not MyoD or myogenin. Extrinsic factors, such as damage or exercise, may activate SCs, i.e. they enter the cell cycle. Activated SCs start to proliferate, creating progeny (the myogenic precursor cells or myoblasts) that express MyoD and MYF5. After proliferation, SCs can either self-renew (maintaining the SC pool), or differentiate into adult myoblasts, initiating differentiation by downregulating Pax7 expression. Finally, the fusion and the terminal differentiation begin with the expression of myogenin and MyoD [4].

In normal, undamaged muscles, 2–7 % of each of the fiber's nuclei are SCs that are mitotically quiescent (expressing Pax7; at G_0 phase); however, as described, when exposed to loading, trauma or injury signals, they activate, proliferate, and either self-renew (maintaining the SC pool), or differentiate into myoblasts that follow terminal differentiation [39]. In order to expand the number of SCs, their withdrawal from quiescence is mandatory. Immunohistochemical (IHC) analysis from the middle region of the vastus lateralis from young men, subjected to one bout of combined endurance and resistance exercises, showed that SCs from both type I and II fibers were able to enter the cell cycle (increased delta-like 1 homolog [DLK1] expression) within 9 h post-exercise [18]. However, although SML may be effective in promoting the activation of SCs, their pool will only increase if they proliferate efficiently. Again, loading through a single bout of voluntary running was able to either promote dynamic changes in rat plantaris muscle messenger RNA (mRNA) expression (increased expression of cell-cycle-related genes 24 h post-exercise) or increased cell proliferation, confirmed by 5-bromo-2-deoxyuridine (BrdU; a thymidine analog nucleoside that labels DNA-replicating nuclei) immunohistochemistry [40]. Another study analyzing the effects of a single bout of high-intensity eccentric exercise showed that after 4 and 8 days the SCs from the vastus lateralis of young sedentary males increased their expression of neural cell adhesion molecule (NCAM) and the fetal antigen 1 (FA1) [41], indicating again that SML was able to potentiate the activation and proliferation of SCs. Curiously, a more recent study was able to detect an increase in both the SC number and expression of cell-cycle-related components (DLK1 for activation, and

proliferating cell nuclear antigen for proliferation), in vastus lateralis muscles of nine young men, only 24 h after an intensive eccentric exercise protocol (300 eccentric knee contractions) [42]. These results suggest a possible fiber-type-specific response to different types and loading intensities. Indeed, data regarding the acute effects of resistance exercise in human skeletal muscle showed an increase in the number of SCs, specifically in the type II fiber type [43]. Nonetheless, others, despite an SC-specific response to the type of contraction mode (concentric or eccentric; SCs increase only upon eccentric contractions), have not described a fiber-type-specific response to acute resistance exercise [44].

Extensive data show that aging specifically reduces the content of SCs in type II skeletal muscle fibers [36, 45]; however, the following studies show that SCs seem to be able to either increase in number or increase mRNA expression of important MRFs following SML. Both situations may be important during SMR. For instance, SCs (expressing NCAM in IHC labeling) from the human vastus lateralis of older adults were able to increase in number 24 h after a single bout of eccentric exercise; however, this increase was clearly diminished when compared with younger men [46]. Moreover, others have also demonstrated that age reduces the ability of vastus lateralis SCs to activate and increase in number following a single bout of resistance exercise, specifically in type II muscle fibers [47]. A more recent study, also using a single bout of resistance exercise, showed that SCs from the vastus lateralis of older adults took longer (72 h) to increase in number, in the type II muscle fibers, when compared with younger men (48 h) [48]. However, an interesting study analyzing the human skeletal muscle response to an unloading/reloading event (skeletal muscle atrophy through limb cast, and loading through cast removal and resistance exercise training), also documented reduced SC activity in older individuals despite no differences being found regarding the mRNA expression of main MRFs [23].

Despite this data collection pointing out the acute activation, proliferation, and increase in the number of SCs promoted by SML, some controversy has been documented in the literature regarding the unloading effects on the number of SCs. Interestingly, a study analyzing the atrophy process using rat hindlimb suspension also indicated, using BrdU staining, that SCs from the gastrocnemius muscle seem to acutely proliferate 6 h after hindlimb suspension, but after 1 week in this condition the number of SCs significantly decreased when compared with weight-bearing controls [49]. Curiously, this increase in the number of SCs during unloading (2 weeks of unilateral whole-leg casting) was also found in human vastus lateralis muscles (on both type I and II fibers) of young males but not in older individuals [23]. Nonetheless, a more recent study analyzing the effects of 2 weeks of a full-leg cast in 12 young men, evinced that the number of SCs did not change despite an increase in myogenin mRNA, which may indicate some activity in the SC pool [50]. Additionally, another study analyzing the effect of neuromuscular electrical stimulation (NMES) on the vastus lateralis of young males subjected to 5 days of muscle unloading (full-leg cast) also showed that the number of SCs did not change; nevertheless, an increase in MyoD and myogenin mRNA may be indicative of the activity of SCs [51]. However, another relevant study analyzing the molecular regulators of the activity of SCs in human vastus lateralis muscles following unloading (2 weeks of full-leg cast) and reloading (exercise after cast removal) also showed that aging deteriorates the ability of SCs to proliferate [52]. All these data regarding the behavior of SCs during unloading suggest different responses between animal and human studies, indicating that methodological frailties or other particularities of this condition (e.g. the underlying cause) may influence the results [53]. Moreover, it is worth mentioning that the discrepancies shown in the different studies might be due to the fact that apoptotic cells in the skeletal muscle promote the fusion of healthy myoblasts, as reported in an animal study by Hochreiter-Hufford et al. [54]. Considering that skeletal muscle atrophy may, per se, induce an acute pro-apoptotic environment both inside and outside myofibers [53], one might consider that this event may transitorily increase myonuclei turnover. The increased mRNA expression of important MRFs may be indicative of this increased SC turnover rate. The reported data clearly support the concept that unloading transiently stimulates the proliferation of SCs; nonetheless, further investigation is warranted to clearly demonstrate its occurrence [55].

In summary, irrespective of the atrophy-related controversy, solid data indicate that SML is an effective way to acutely increase the activation, proliferation, and number of SCs, enabling a transient increase in BMP.

2.1.2 *Chronic Effects*

Considering the acute SML effects in the SC pool, it seems appealing to explore their long-term consequences to determine whether the more active musculature, the greater its number of SCs, and therefore the greater its BMP.

Again, several chronic SML conditions seem to effectively maintain the elevated number of SCs [37, 43, 56–62]. Nevertheless, debate continues regarding the requirement of the presence and fusion of SCs during muscle hypertrophy [63] and regrowth following atrophy [64]. For instance, an animal study analyzing the mechanical overload effect in SC-depleted plantaris muscle showed that the

addition of SCs is not necessary for muscle hypertrophy [65]. Moreover, the muscle regrowth capacity, following atrophy (hindlimb suspension), in SC-depleted mice soleus was also independent of the presence of SCs [64]. These animal studies simply suggest that the myonuclear domain is dynamic. Solid data indicate that the acquisition of new myonuclei for further hypertrophy is only mandatory when the myonuclear domain size exceeds a certain threshold [66]. Curiously, both animal studies showed a significant reduction in muscle turnover (BrdU-positive myonuclei) of SC-depleted muscles [64, 65]. Additionally, a recent study analyzing the role of human muscle SCs during 6 weeks of aerobic interval training (nonhypertrophic stimulus) demonstrated that SCs intensely contribute to muscle remodeling [38]. Considering this, one can consider that the depletion of muscle SCs and therefore the loss of myonuclei turnover and further hypertrophy, may be decisive for a proper SMReg, i.e. to maintain muscle tissue structure and functionality during SMR, as has already been mentioned [7, 22].

Despite this controversy, a recent study analyzing the effect of 16 weeks of resistance exercise showed that SCs from the quadriceps muscles increased in number in both type I and II fibers. Additionally, a correlation between the relative changes in the number of SCs and the percentage increase in the lean muscle mass was observed [43]. Interestingly, a study using cluster analysis to evaluate the relationship between myofiber hypertrophy and SC pool in the vastus lateralis of 66 humans showed that those who had larger SC pools in basal conditions had greater capacity to increase SC number, to increase fiber nuclei addition and to achieve greater fiber hypertrophy in response to 16 weeks of knee extensor resistance training [37]. These results indicate the importance of a larger SC pool during a stressful event such as resistance exercise. However, reduced basal SC pools, such as those seen in type II fibers of elderly human skeletal muscles, are also able to increase their number of SCs (and their fiber size) in response to 12 weeks of resistance exercise [36]. Therefore, the association between the content of basal SCs and muscle fiber hypertrophy following resistance exercise continues to be debatable.

Another study analyzing the effect of 10-week resistance training in 18 women with trapezius myalgia also showed an increase in the number of SCs in both type I and II muscle fibers, conjugated with an increase in SCs expressing Ki-67 (indication of cell cycle activity), demonstrating enhanced proliferation [60]. This type of chronic SC pool adaptation to resistance training has been previously described in nine adult women in the same muscle group, but without myalgia [56]. Remarkably, even during a 12-week, light-loading resistance training, SCs from vastus lateralis of 12 young men were able to increase their pool [61], suggesting that SML can be an interesting way to increase the BMP in situations where solid resistance training is not possible, e.g. during moderate skeletal muscle injuries or to prevent atrophy during limb casting. In addition, 12 weeks of aerobic training (three sessions of 45 min per week at 70 % heart rate reserve) performed by sedentary adults (6 males and 17 females) resulted in increased content of SCs in type I fibers (but not type II fibers) [62], indicating again that a fiber-type-specific response and a less demanding type of SML seem to be effective in enhancing the muscle BMP.

As described, some studies show that the capacity of SCs to respond to acute SML seems to be preserved during aging, mainly in type I muscle fibers. Likewise, the SC pool of both elderly men and women maintains its ability to increase during both endurance [57, 67] and resistance training [59, 67, 68]. A study analyzing the effect of 14 weeks of intermittent endurance cycling (45 min daily, 4 days per week) in the vastus lateralis of 11 men (aged 70–80 years) showed that the SC (NCAM-positive) pool effectively increases [57]. Similarly, the SC (NCAM-positive) pool of vastus lateralis of 13 older men (average 72 years of age) increased following 12 weeks of resistance training, wherein a dramatic fiber type II-specific response occurred [59]. Additionally, both elderly men and women were also able to increase their number of SCs in type II muscle fibers from the vastus lateralis in response to 6 months of resistance training [68]. Finally, a study specifically analyzing both lower body endurance (intermittent cycling) and upper body resistance (three sets of three strength-training exercises) SML potential to increase the SC pool in elderly men over a 14-week period (three times per week) revealed that simultaneous lower body endurance and upper body resistance training effectively increased the SC pool of the vastus lateralis and deltoid muscles, respectively, solely in type II muscle fibers [67]. Curiously, endurance training (6 months) in obese male type 2 diabetes patients (61 $\pm$ 6 years) did not change the content of the SCs in the vastus lateralis muscle [69]. One can consider that either the condition or the training intensity (much lower than that used by others [57, 67]) possibly blunted muscle growth and response of the SCs.

Once again, these results demonstrate the SML potential to mitigate the specific age-dependent decline of SCs [36], specifically seen in type II muscle fibers [45].

Finally, it is important to acknowledge that the SC pool is highly plastic, i.e. it proficiently adapts to SML but also adapts to unloading, as reported in a study analyzing the SC pool from the vastus lateralis of 15 young men who, despite an increase in SCs due to 90 days of resistance training, their number of SCs returned to pre-training values within 90 days of detraining [58]. This study also indicated that only 3 days after detraining, SCs seemed to

suddenly decrease their proliferation capacity, as suggested from the mRNA values of the cell-cycle marker p21.

In summary, these studies collectively indicate that SML seems to ensure a greater BMP that is maintained in the active musculature, even throughout the aging process. This important feature may be a determinant in the success of SMR.

2.1.3 Satellite Cell Niche Modulation

The microenvironment (i.e. their surroundings and everything within, contacting and influencing stem cell behavior) in which the stem cells reside is their niche [70, 71], and although there are many unanswered questions regarding the characteristics of this milieu, like its molecular regulatory aspects on different organs, it is becoming apparent that in the skeletal muscle this microenvironment plays a major role in the fate of SCs [72]. The behavior of SCs can be severely altered by growth factors, cytokines, and other diffusible molecules produced by adjoining cells and surrounding ECM [72, 73]. Briefly, as described, molecules from the immediate niche and the local or systemic milieu of the SCs, may alter their state. SCs seem to (1) activate, in an autocrine-fashion, upon contact with HGF and epidermal growth factor (EGF), from SCs, myofibers, and interstitial cells, or present in serum in the immediate niche and nitric oxide ($^{\cdot}$NO), a diffusible molecule (produced by diverse cell types, including epithelial cells, endothelial cells, fibroblasts, macrophages and muscle cells) from the systemic niche; (2) proliferate with FGF, IGF-1, tumor necrosis factor (TNF)-like weak inducer apoptosis (TWEAK) and delta-1 protein, a Notch ligand; and (3) differentiate with IGF-1. Their quiescence is maintained mostly in the presence of calcitonin and laminin. The main functional inhibitors are myostatin (activation and self-renewal), BDNF, TWEAK, and FGF (differentiation). The Wnt proteins affect the fate of the cell; the SDF-1 (or CXCL12) acts in the migration process, and the integrin $\alpha 4\beta 1$ (VLA4) acts in the myoblast fusion [4, 74]. These intricate interactions between these different molecules and growth factors, which can be modulated during diverse physiological or pathological situations, unmask the niche complexity that governs the fate of SCs, and therefore it seems important to unveil the modulatory effects of SML.

Recently, data addressing which myokines seem to be produced and released by the contracting skeletal muscle (postulating a conceptual foundation that emphasizes the skeletal muscle as a 'secretory organ' capable of communicating and inducing effects in an autocrine, paracrine, or endocrine fashion) showed that some myokines (IL-6, BDNF, IL-7, and IL-15) might exert specific actions on the proliferation and differentiation of SCs [75]. Nonetheless, SML effects are broader with respect to changing the niche molecular characteristics of SCs since they seem to influence the production, expression, and release of different mediators in different tissue cells [75].

As described, in addition to SCs, SMReg may include stem cells from other tissues, such as circulating endothelial progenitor cells (EPCs) and circulating bone marrow progenitor cells (cBMPs) that effectively recognize, migrate, and contribute to the SC pool, and regenerate injured skeletal muscle [3–5]. Curiously, when comparing amateur runners with sedentary controls, the number of cBMPs ($CD34^+$, $CD38^+$, $CD33^+$) was three- to fourfold higher in runners [76]. Additionally, regarding other stem cells with myogenic potency, i.e. the EPCs or blood vessel stem cells ($CD34^+$, $CD133^+$, KDR^+), SML seems to be a determinant in increasing their numbers [77]. These two examples support the likely capacity that SML seems to have on increasing the availability of stem cells, which, along with the migration capacities, also have myogenic potency that can be important during SMR.

The likely skeletal muscle chemotactic capacity seems to be supported by its own myokines and other chemical mediators produced by the surrounding tissue cells, as shown by one study, using transgenic mice, genetically marked cells, cell cultures, and immunostained human muscle, evaluating the relationship between the EPCs and the niches of the SCs [78]. Its main findings were that SCs were markedly associated with capillaries, their numbers correlated with muscle capillarization, and both stem cell groups seemed to communicate in a paracrine faction through the release of VEGF, IGF-1, HGF, and FGF, reciprocally promoting angiogenesis and the proliferation of SCs, mainly through VEGF production of SCs, and culminating in a spatiotemporal relationship between myogenesis and angiogenesis [78]. Irrespective of the VEGF central role in angiogenesis and which were the main cells expressing it, it is clear that SML increases the number of muscle capillaries and VEGF expression [79], and this niche alteration, together with the proliferation effect of SCs, provided a possible means of achieving an improved SMR. Actually, as already demonstrated, VEGF infusion in regenerating mice tibialis anterior muscles not only promotes proper SMReg but also protects SCs from apoptosis [80].

Finally, because interactions between SML, SMReg and IGFs have been recently reviewed [81], this issue will not be addressed further. Nonetheless, as reviewed, since SML increases IGF-1 expression and this growth factor enhances SMReg [82], it is reasonable to consider SML as a positive SMR modulator.

3 SML and Skeletal Muscle Repair

Considering that SML effectively increases skeletal muscle myogenic potential, i.e., induces SCs pool growth, in both acute and chronic conditions, it seems appealing to

scrutinize its therapeutic and prophylactic effects during SMR. Nonetheless, as described, SMR characteristics and outcomes depend upon the type of muscle injuries that are highly heterogenic in severity, making human studies scarce.

3.1 SML Therapeutic Effects

Upon muscle injury, the most common acute treatment adheres to the RICE procedure. This widely used approach, using immobilization as the fundamental principle, attempts to acutely reduce blood flow and edema, minimizing inflammation and, afterwards, gradual increases in SML (within pain restrictions), as well as other therapeutic options (medication, ultrasound, and hyperbaric oxygen therapy), are recommended [15]. Interestingly, growing data indicate a crucial role for inflammation in achieving proper SMReg [83], and heat application may accelerate the proliferation and differentiation of SCs [84]. Consequently, despite the acute benefits of RICE in controlling further tissue damage, it seems relevant to acknowledge that if the forced immobilization continues, it will ablate the potential of SML to increase the activation and proliferation of SCs, and possibly impair SMR.

In the particular case of hamstring injuries, the lack of studies analyzing different types of rehabilitation protocols shows that common practice is based on empirical knowledge and cannot either be favored or disproved [85]. However, two recent studies showed that rehabilitation protocols accentuating active eccentric exercises during recovery from hamstring injuries are more effective than those with usual protocols, i.e., with less emphasis on eccentric SML [86, 87]. These results may elucidate the importance of a more intense SML protocol when compared with protocols emphasizing flexibility exercises. An interesting animal study analyzing the adjuvant effect of SML on muscle-derived stem cell (MDSC) transplantation during SMR might elucidate a possible explanation for these outcomes [88]. The results of this study showed that in addition to an increased proliferation on mechanically-stimulated MDSCs, SML (5 weeks of treadmill running) enhanced MDSC transplantation after injury, prevented fibrosis accretion, and increased skeletal muscle vascularity [88]. Curiously, a former study analyzing marked bone marrow cells transplanted into mice that were submitted to both forced running exercise (downhill running) and EDL overload (surgical tibialis anterior removal) also showed an increased incorporation of bone marrow-derived cells into skeletal muscle [89]. Both studies suggested that in addition to the mentioned increase in the SC pool, SML may be an effective way of enhancing SMR by stem cell chemotactic attraction. In addition, during SMR following notexin injury in the soleus muscles of female rats, recovery of muscle mass and decreased fibrosis was also shown in active (caged with access to running wheel and performing treadmill running 5 days/week) compared with sedentary (kept in normal cages) animals [17]. This study also showed that active rats recovered their muscle mass to pre-injury values within 21 days of post-injury whereas sedentary rats failed to restore muscle mass to pre-injury values even after 42 days. Moreover, SCs from active rats had better and faster proliferation (measured by proliferator cell nuclear antigen and MyoD) and increased differentiation (higher expression of myogenin) [17]. Decreases in fibrosis accretion also occurred using stretching exercises (passive mobilization) in the first 2, 7, and 14 days after laceration injury in the gastrocnemius muscle of male rats [90]. Interestingly, despite the lack of studies regarding SML influence in ECM dynamics during SMR, it is known that both collagen production and MMP (proteases that mainly degrade ECM collagen) activity acutely increase with loading and, chronically, SML increases both collagen content and turnover [13]. More recently, a study analyzing the acute and chronic effects of endurance training (45 min/10 days/5 weeks) in the vastus lateralis of ten men, showed acute (2 h after exercise bout) increases in MMP-9 (modulates collagen turnover and growth factor availability) activity and chronic (10 days of training) increases in MMP-2 (cleaves ECM and basal lamina in angiogenesis and is implicated in successful SMR) and tissue inhibitor of MMP-1 mRNA expression [91]. This ECM enzymatic modulation may determine the favorable, less fibrotic, skeletal muscle phenotype seen in loaded muscle during SMR.

Finally, as described, SMR is also influenced by an important contribution of the inflammatory system. Curiously, increases in macrophage content during SML (10 weeks of resistance training in 18 women identified as having trapezius myalgia) [60], have been reported. Furthermore, improved phagocytic activity in macrophages from rats submitted to 1 h of swimming [92] may demonstrate a possible acute improvement of SML on macrophage function that may also be important for removing cellular debris during SMR. Despite the fact that some macrophage functions (antigen presentation, major histocompatibility complex II expression and antiviral activity) may be hindered by SML, others, such as tumor cytotoxicity, chemotaxis, and phagocytosis functions, are enhanced by both acute and chronic SML [93]. Nonetheless, many aspects regarding the interaction between the cellular innate immune function and exercise continue to be unknown [94].

3.2 SML Prophylactic Effects

As elucidated, acute SML compels SCs to activate and proliferate, and these effects possibly justify the increased

number of SCs in muscles loaded chronically. Thus, one could speculate that SML effectively increases the BMP of muscles. It is therefore plausible that this theoretical prophylactic effect may be significant in medical conditions that either force muscle unloading (such as limb casting) or damage skeletal muscle (surgery). Disregarding the accidental injuries, this prophylactic effect should be considered in programmed interventions that imply skeletal muscle unloading or injury. Evidence in animal studies indicates that NMES may prevent loss of myonuclei and SCs by apoptosis, preserving the SC pool for further SMR, and attenuate decreases in muscle size and force production [95, 96]. Similar results were also found in young human skeletal muscle, subjected to disuse atrophy (5 days of one-leg casting), with or without NMES sessions. Their data showed that NMES prevented loss of muscle mass and mRNA expression of important regulators of muscle protein breakdown, but neither preserved muscle strength nor altered the number of myonuclei and SCs [51]. Nonetheless, during conditions characterized by impaired limb movement, electrical stimulation should be considered since it may be a potential strategy to avoid loss of muscle mass.

4 Conclusion and Further Perspectives

This review highlights the possible role of SML in increasing the myogenic potential that possibly drives the SMR toward a more functional phenotype. The data presented also evince the SML therapeutic effect upon injury, increasing and stimulating proper SMReg and inhibiting excessive fibrotic deposition. Thus, SML seems to promote (1) acute and chronic increases in the number of SCs; (2) increased activation, proliferation, and terminal differentiation of SCs; (3) increased migration of other myogenic stem cells; (4) increased angiogenesis; (5) inhibition of excessive fibrosis deposition during SMR; and, collectively, (6) a faster and proficient SMReg. Moreover, SML efficiently promotes milieu alterations through myokines released by myofibers upon contraction, which induce the production and release of cytokines from surrounding cells. This milieu molecular alteration may support the muscular chemotactic activity that increases stem cell migration during SMR, and promote a chemical environment more suitable to successfully react upon injury. Other important, but less clear, SML features may be hidden in the physical and mechanical force-related imposition, upon contraction, to all cells within the skeletal muscle and its ECM. Possibly, this physical cellular stress, perceived by all cells, is as effective in altering cellular function as the chemical and molecular milieu alterations.

This data collection suggests that active skeletal muscles might be better prepared to effectively respond to a muscle injury. This prophylactic effect should be contemplated in clinical situations where muscle atrophy or injury intentionally occurs. This review also evinces the lack of studies analyzing the cellular and molecular alterations promoted by SML during SMR, particularly regarding the ECM alterations. Finally, we seek to encourage physical therapists, sports medicine specialists, and muscle physiologists to deliberately promote, as soon as possible, a more active therapeutic approach to SMR.

Compliance with Ethical Standards

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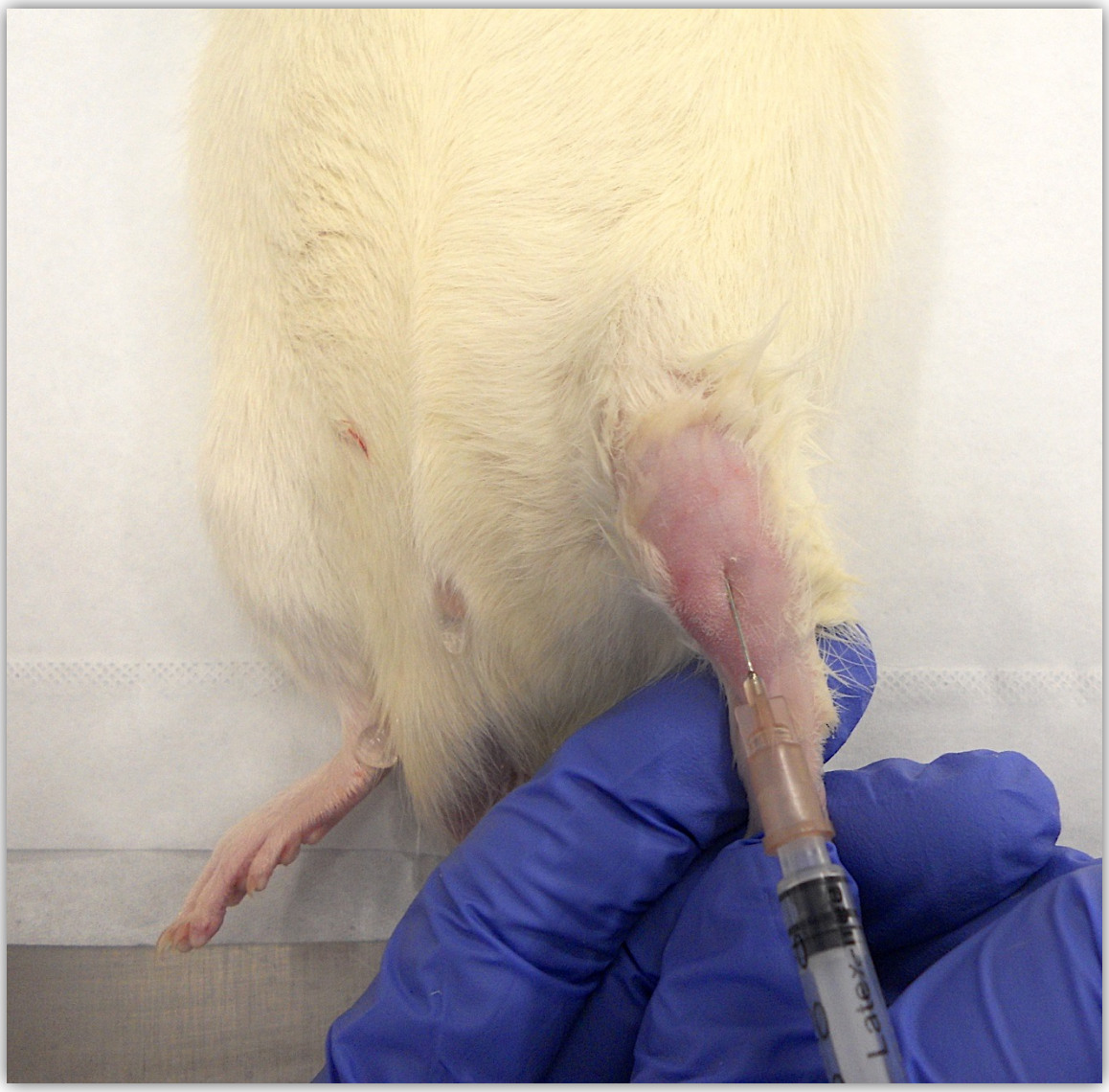
3. EXPERIMENTAL STUDIES



[Study 1]

Detrimental effects of sedentary behaviour on rat skeletal muscle healing after cardiotoxin-induced damage

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Keywords:	injury, regeneration, fibrosis, histology, repair

Detrimental effects of sedentary behaviour on rat skeletal muscle healing after cardiotoxin-induced damage

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ABSTRACT

The aim of this study was to test the hypothesis that sedentary behaviour (SB) impairs male Wistar rat skeletal muscle repair (SMR) after cardiotoxin (CTX)-induced damage. Individually caged animals spent 8 weeks either as a sedentary group (SED, n=20) – without access to a running wheel – or as a control group (EX, n=20) – with access to a running wheel for voluntary running. Afterwards, all rats had each tibial anterior muscles injected, either with CTX (CTX; right muscle) or saline solution (Sham; left muscle) and were sacrificed (n=5 per group) on the 1st, 3rd, 7th and 15th day post-injection (dpi). Non-overlapping images taken from the muscle injected area were semi-quantitatively analysed to calculate the level of muscle damage. The SMR was measured by quantifying the number of regenerating fibres (myotubes), their cross-sectional area (CSA) and the fibrotic tissue (FT) accretion. CTX-induced damage was similar in both SED and EX groups at 1st dpi. Regarding

SMR, SED CTX muscles showed decreased myotubes CSA and their number remained increased when compared to EX group on the 15th dpi ($p<0.01$), both indicative of SMR delay. Moreover, on both the 7th and 15th dpi, SED CTX muscles presented more FT accretion than EX ($p<0.01$). The presented data support our hypothesis that SB delays SMR and increases FT accretion, promoting a worst skeletal muscle phenotypic adaptation upon injury.

Keywords: injury, regeneration, fibrosis, histology, repair

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INTRODUCTION

Sedentary behaviour (SB) is a risk factor for multiple organ dysfunction, particularly to skeletal muscle (Pedersen, 2009). Indeed, the lack of physical activity deeply affects the skeletal muscle tissue phenotype, its morphology and function, e.g., promoting skeletal muscle atrophy and loss of strength (Booth, et al., 2012). Besides these overall detrimental effects, less is known about the possible interaction between SB and the skeletal muscle repair (SMR) process.

The SMR is a highly complex process involving the coordinated recruitment of a wide range of cells and a meticulous interactions with the inflammatory system that culminates either with the development of: (1) a favourable phenotype – proper repair of damaged skeletal muscle fibres by their replacement with new ones, not by fat or fibrosis, with functional maintenance; or (2) an unfavourable phenotype – hindered SMR with substitution of muscle fibres by fat or fibrosis, with scar tissue formation and functional impairment (Moyer and Wagner, 2011). In fact, SMR has a pivotal function in order to appropriately cope with different insults (like exercise, immobilization or injury) and it is known that this organ's regenerative capacity relies on the presence and function of a myogenic stem cell population known as satellite cells (SCs) (Otto, et al., 2009). Indeed, we have recently proposed a skeletal muscle loading prophylactic effect, i.e., a possible beneficial effect of increased skeletal muscle activity in its regenerative capacity since increased physical activity and/or exercise appears to be beneficial in improving skeletal muscle regeneration and impairing fibrotic tissue (FT) deposition (Teixeira and Duarte, 2016).

Considering that SMR may be affected by several factors (Ambrosio, et al., 2009) and the detrimental effects of SB on muscle phenotype, it seems relevant to adequately address if sustained SB would affect SMR ability upon acute injury. Therefore, the aim of this experiment was to test the hypothesis that SB impairs SMR, determining if 8 weeks of SB alters the SMR process of tibial anterior muscle in response to a cardiotoxin (CTX)-induced damage.

MATERIAL AND METHODS

Animals and experimental design

After one week of quarantine, 40 male Wistar rats with 2 months old (Charles River Laboratories, Barcelona, Spain) were arbitrarily assigned to either a sedentary group (SED, n=20) or an exercise control group (EX, n=20). All animals were kept in a controlled environment, with a constant humidity of 50-60% and temperature of 22±2 °C, in an inverted 12h light/dark cycle. Standard rat chow and tape water were provided ad libitum during the entire experimental period. EX rats were housed individually in cages with overall dimensions of 480x315x470 mm^3, equipped with a complete activity wheel system (Tecniplast 2154F0105) that allowed them to run voluntarily. SED rats were also housed individually in cages with similar dimensions but without the activity wheel system. Following 8 weeks in these conditions each animal, under light anaesthesia induced by ketamine and xylazine intraperitoneally (ip), was injected with 30 µl of 20 µM cardiotoxin (CTX; Naja mossambica mossambica; C9759, Sigma-Aldrich) in the mid belly of the right tibial anterior muscle (CTX muscle) and with 30 µl of saline solution (Sham; 0.9% of NaCl) in the same muscle region of the left tibial anterior muscle (Sham muscle).

CTX was prepared by dissolving a recently opened container in PBS. The fresh solution was divided into Eppendorf tubes as 10 ml aliquot stocks, flash frozen, and stored at -80 ºC. Each tube was defrosted fresh before injection and not re-used. Injections where performed using a 50 µl, 22-gauge needle-fixed syringe (Hamilton; 20736, Sigma-Aldrich) and in order to maintain the experimental procedure homogeneity, i.e., ensuring that similar injuries were induced to all experimental groups, all the injections were made by the same researcher. After these procedures, all animals were kept in the same conditions and 5 animals from SED and EX groups were sacrificed on the 1st, 3rd, 7th and 15th day post-injury (dpi). Total distance ran by each rat of EX group was documented daily. The study was approved by the ethics committee of the Faculty of Sport from the University of Porto. All the institutional guidelines for the care and use of animals have been followed.

Tissue harvesting and processing for light microscopy

All animals were sacrificed by a lethal ip injection of ketamine and xylazine and the tibial anterior muscles excised, cleaned of adjacent soft and fat tissue, cut in half in the mid belly region and fixated in a solution containing 4% paraformaldehyde (P6148; Sigma-Aldrich), 2,5% sucrose (S0389; Sigma-Aldrich) and 0,1% glutaraldehyde (G5882; Sigma-Aldrich) in PBS (pH 7.2) at 4ºC for 24 hours. After fixation, both halves were dehydrated through graded ethanol solutions, cleared in xylene and embedded in paraffin blocks. Transverse 5 µm thick sections from each muscle part were cut on a Leica 2125 rotary microtome (Leica Microsystems). The produced slides were used for histomorphological evaluation and fibrous tissue accretion analysis.

Muscle Histomorphometry and Evaluation of Fibrotic Tissue Accretion

Muscle sections were stained with haematoxylin and eosin (H&E), analysed in a light microscope (Axio Imager A1, Carl Zeiss; Germany) with a 40x magnification objective and images photographed by an attached digital camera with Axio Vision 4.7 software. Acquired non-overlapping images were then examined with ImageJ software (NIH, Bethesda, MD) to quantify the morphological features of the muscles sections. For consistency, the histomorphological evaluation was performed on multiple single frame images (6 to 9 images obtained per section) taken from the same muscle section (6 different sections analysed per muscle) and, exclusively, within the damaged area, i.e., muscle area with clear inflammatory infiltrate, presence of necrotic fibres (degenerating fibres with fragmented sarcoplasm), regenerating fibres (small basophilic and central nucleated fibres with normal sarcoplasm) and excessive FT accretion.

Muscle damage

Muscle damage was calculated by a semi-quantitative histomorphological evaluation performed through a previously described procedure (Dinis-Oliveira, et al., 2007), here adapted to the skeletal muscle tissue. The subsequent parameters analysed in, at least, between 36 to 54 images of each muscle in a blind fashion, allowed a semi-quantification of: 1) the cellular degeneration; 2) the inflammatory activity; 3) the necrosis extent and 4) the tissue disorganization. Cellular degeneration (i.e., fibres dilatation, sarcoplasm vacuolization and density) was scored per percentage of the affected tissue:

score 0 = no change from normal; score 1 = a limited number of isolated cells (up to 5% of the total cell number); score 2 = groups of cells (5 to 30% of total cell number); score 3 = diffuse cell damage (more than 30% of total cell number). The severity of fibre necrosis was scored as: grade 0 = without necrosis; grade 1 = dispersed necrotic foci; grade 2 = confluent necrotic area; grade 3 = massive necrotic area. Inflammatory activity was scored as: grade 0 = no cellular infiltration; grade 1 = mild leukocyte infiltration (1 to 3 cells per image); grade 2 = moderate infiltration (4 to 6 leukocytes per visual field); grade 3 = heavy infiltration by leukocytes. Tissue disorganization was scored as: grade 0 = normal structure; score 1 = less than one-third of the image; score 2 = greater than one-third and less than two-thirds of the image; score 3 = greater than two-thirds of the image. Finally, all the previous parameters were summed to produce a total damage score that summarized the overall histological features. Percentage of necrotic fibres on the 1st dpi was also calculated from the total number of analysed cells in each image.

Skeletal muscle repair

The SMR progress was characterized by measuring the myotubes cross-sectional area (CSA), their percentage and the FT accretion at each dpi. Small basophilic and central nucleated fibres, with normal sarcoplasm were accounted as myotubes. Myotubes CSA was defined by their individual fibre size (μm^2). Percentage of myotubes and normal fibres (undamaged fibres with peripheral nuclei, intact sarcolemma and non-fragmented sarcoplasm) was determined from the total number of analysed cells in each image. We have also evaluated myotubes circularity ($4\ \pi$ [Area]/[Perimeter]^2) a shape

descriptor that indicates a perfect circle when the value is 1 and an increasingly elongated shape as the value approaches 0,0. For the quantification of fibrotic tissue area, the picrosirius red (SR) method (Sweat, et al., 1964) was used to stain the muscle sections. Briefly, slides were incubated on 0,1% SR in saturated picric acid for 1h, rinsed in 0,5 % acetic acid, dehydrated in ethanol and cleared in xylene. This method stains collagen tissue bright red and muscle tissue yellow. Again, 6 to 9 non-overlapping single frame images, taken from the same muscle section and within the damaged area were analysed with Image-Pro Plus 6.0 software (Media Cybernetics). The area covered by FT was quantified in all experimental groups.

Statistical analyses

The Kolmogorov-Smirnov test was used to determine within-group normality for a given variable and the Levene´s test to determine homogeneity of variance. Normal distributed variables are presented as mean ± standard deviation (SD). Nonetheless, most of the variables lacked normal distribution and, therefore, differences between groups and days within group were tested with the Mann-Whithney non-parametric test (two-tailed) and Kruskal-Wallis test, respectively._The Chi-Square test was used to test the bivariate analysis. These variables are presented as median and interquartile range (IQR: 1^{st} – 3^{rd} quartiles). All statistical analyses were performed using SPSS, version 21. Differences were considered significant at $p<0.05$.

RESULTS

Voluntary physical activity and body weight

At the end of the 8 weeks, EX group ran on average 21.02±12.05 km/week. EX body weight was significantly lower than SED (384.15±26.79g vs. 403.47±33.17g, respectively, $p<0.01$).

Overall Histomorphological data

CTX injections efficiently promoted marked muscle damage when compared to Sham muscles. Since Sham muscles presented very limited muscle damage (only through the needle mechanical injury) culminating in minor histological changes, their data were not considered for this articles' discussion. Muscle damage semi-quantitative data of all groups are depicted in table 1. Representative images of tibial anterior muscles sections, stained with H&E from the 1st and 3rd dpi, are illustrated in figure 1, and from the 7th and 15th dpi in figure 2. The myotubes CSA, observed in all groups, is depicted in figure 3. Representative images of tibial anterior muscles sections, stained with SR, and FT area are illustrated in figure 4 and 5, respectively. For a better understanding of the muscle damage, SMR and FT temporal evolution, their results are presented at each dpi.

1st day post-injury

No differences were found in muscle damage data between SED and EX groups. Both groups showed the highest classification on the cellular degeneration, inflammatory activity, necrosis extent and tissue disorganization scores (figure 1, table 1). Additionally, both groups had similar percentages of

necrotic fibres, (87% in EX vs. 90% in SED; p>0.05). As expected, at this time point, myotubes were absent. The median FT area (figure 5) was 10.3 (5.7 – 15.8) mm^2 and 11.2 (9.8 – 13.3) mm^2 for SED and EX groups, respectively (p>0.05). Moreover, there were no differences in FT area comparing CTX with Sham muscles (p>0.05).

3rd day post-injury

EX group showed higher levels of cellular degeneration, necrosis and tissue disorganization than SED group (p<0.05), resulting in a higher total damage score compared to SED group (p<0.05) as illustrated in figure 1 and table 1. The median FT area (figure 5) was 9.7 (6.5 – 12.5) mm^2 and 11.7 (8.5 – 14.4) mm^2 for SED and EX groups, respectively (p>0.05). Again, there were no differences in FT area comparing CTX with Sham muscles (p>0.05).

7th day post-injury

Despite no differences on the overall muscle damage score, SED group showed more cellular degeneration, necrosis and tissue disorganization than EX group (p<0.05) as illustrated in figure 2 and table 1. Interestingly, SED group presented a significantly higher myotubes CSA (figure 3) compared to EX group (p<0.01). Regarding the percentage of myotubes, SED group presented a decreased value compared to EX group (54,8% vs. 62,9%, respectively, p<0.01). The median muscle fibres circularity was 0.77 (0.67 – 0.86) and 0.84 (0.75 – 0.89) for SED and EX groups, respectively (p<0.01). Regarding FT accretion (figure 4 and 5), the median FT area was 35.4 (29.9 – 42.4) mm^2 and 25.9 (21.7 – 28.5) mm^2 for SED and EX groups, respectively

(p<0.01). Both EX and SED CTX muscles showed increased FT accretion compared to Sham muscles (p<0.01).

15th day post-injury

No differences were found in total damage score between SED and EX groups. Despite some minor inflammatory activity observed in SED group, both groups presented the lowest levels on the cellular degeneration, necrosis extent and tissue disorganization scores (table 1). As demonstrated in figure 3, the median myotubes CSA was 329.3 (193.9 – 513.9) μm^2 and 590.9 (419.3 – 830.7) μm^2 for SED and EX groups, respectively (p<0.01). Regarding the percentage of myotubes, SED group presented an increased value compared to EX group (43% vs. 11.3%, respectively, p<0.01). The median muscle fibres circularity was 0.63 (0.54 – 0.71) and 0.58 (0.51 – 0.65) for SED and EX groups, respectively (p<0.01). The median FT area (figure 5) was 18.1 (15.3 – 21.2) mm^2 and 8 (6.3 – 14) mm^2 for SED and EX groups, respectively (p<0.01). Moreover, only FT area from SED CTX muscles remained increased when compared to Sham muscles (p<0.01).

DISCUSSION

Our results demonstrate that (1) CTX-injections on the tibial anterior muscle efficiently promoted a similar degree of muscle damage on both EX and SED CTX muscles when compared to saline-injection Sham muscles, and (2) the SMR process was hindered in SED compared to EX group, as evidenced by the myotubes features and FT accretion.

On the 1st dpi, CTX-induced damage was similar between SED and EX groups that presented the highest scores in all the evaluated parameters. Additionally, no differences were found in the percentage of necrotic fibres, demonstrating that neither SB nor regular voluntary running change the susceptibility to CTX effects and, more importantly, that the experimental procedure was homogenous between groups. It is relevant to acknowledge that skeletal muscle CTX injection, a commonly used method in the literature to produce local damage (Lepper, et al., 2009, Sousa-Victor, et al., 2014), induces a potent and irreversible muscle contracture, possibly through major membrane-calcium imbalances (Lin Shiau, et al., 1976). Besides this direct toxic effect of CTX to muscle fibres, it seems appealing to consider the contribution of an impaired blood flow caused by the continued muscle contraction (Wisnes and Kirkebo, 1976) to the physiopathology of CTX-induced damage. As expected, Sham muscles presented very limited signs of muscle damage, which are justified by the needle mechanical injury, and therefore, data from their minor histological changes were disregarded because did not relate to the objective of this article. It must be noted that SMR features were not detected at this time point, mainly explain by the short period of time between muscle injection and animal sacrifice.

Curiously, on the 3rd dpi, EX group showed increased muscle damage (higher cellular degeneration, necrosis and tissue disorganization scores) compared to SED group ($p<0.05$). These results show that enhanced muscle voluntary recruitment following an acute muscle damage increases its susceptibility to further deterioration, which might be explained by 1) the mechanical strain applied to the already damaged sarcolemma and myofibrils, and 2) the

metabolic overload, specifically associated with calcium-related changes (Mikkelsen, et al., 2004, Orrenius, et al., 2003). Despite no differences in the FT accretion, signs of an early SMR process were observed in both groups (figure 1), i.e., increased number of myotubes. However, myotubes CSA was not possible to measure accurately at this time point because of their small size and great density.

On the 7th dpi, no differences were found on the overall muscle damage score, however, opposite to 3rd dpi, SED group presented higher scores in cellular degeneration, necrosis and tissue disorganization compared to EX group ($p<0.05$), suggesting an impaired tissue resolution, i.e., a prolonged accumulation of degenerated and/or necrotic cells and debris. Interestingly, SED group showed significantly higher myotubes CSA compared to EX group ($p<0.01$) suggesting that SB may exert a putative beneficial effect in the myotubes development. Nevertheless, SED group also showed a significantly lower myotubes circularity compared to EX group ($p<0.01$), suggestive of a SB-related mechanical tension defect. Indeed, the lower mechanical tension might promote the development of an irregular myotubes longitudinal alignment i.e., with lower circularity and, therefore, increased myotubes CSA, as suggested in figure 2. Others have also documented that fibres with circular shape are characteristic of regenerating fibres (Schmalbruch, 1990). Moreover, SED group presented lower myotubes percentage compared to EX group, suggesting that SB delays and impairs the satellite cells activation, proliferation and differentiation processes upon injury, contrary to the faster and greater myotubes formation rate promoted by regular voluntary running. Interestingly, opposite effects of those seen on the 3rd dpi, regarding

metabolic overload through calcium-related changes, might now explain this enhanced myoblast proliferation, differentiation and maturation capacities in EX group (Tu, et al., 2016). Moreover, data from FT area (figure 5) indicates that EX group developed a favourable phenotype, i.e., with diminished FT accretion compared to SED group ($p<0.01$), and that both EX and SED groups increased their FT accretion when compared to Sham muscles ($p<0.01$). Regardless of the indispensable role of the typically reversible collagen deposition during SMR (Serrano, et al., 2011), excessive or dysregulated fibrotic response upon injury is a common pathological outcome of many diseases (Wynn and Ramalingam, 2012). Interestingly, despite the lack of studies regarding voluntary running and exercise influence in ECM dynamics during SMR, it is known that they have effects on both collagen turnover and matrix metalloproteinases (proteases that mainly degrade ECM collagen) activity (Kjaer, et al., 2006). Yet, others also indicated that enhanced muscle recruitment, through passive mobilization, effectively reduce FT accretion during SMR (Hwang, et al., 2006). Again, SB might be disturbing the fibroblast response to injury, inducing excessive FT synthesis and promoting the development of a more dysfunctional skeletal muscle phenotype upon injury.

Finally, on the 15th dpi, no differences were found on the overall muscle damage scores and both groups showed the lowest scores in all the evaluated parameters, apart the minor inflammatory activity detected in SED group ($p>0.05$). Opposite to data on 7th dpi, SED group showed significantly lower myotubes CSA compared to EX group ($p<0.01$), clearly indicating the SB deleterious effects on myoblasts maturation and growth during SMR and

substantiating the favourable effects of increased voluntary running in promoting a faster muscle mass recovery. Similar results, addressing the positive effects of enhanced muscle recruitment, through electrical stimulation, on rat skeletal muscle histomorphometry, were recently documented (Zissler, et al., 2017), and some human studies also indicated that applying greater muscle mechanical tensions, through eccentric exercise training, has marked effects on the development of a favourable skeletal muscle phenotype and functional maintenance during SMR (Askling, et al., 2014, Askling, et al., 2013). Moreover, at 15th dpi SED group presented increased myotubes percentage compared to EX group (43% vs. 11.3%, respectively, $p<0.01$), i.e., SED group presented only 57% of normal undamaged fibres compared to 89% of normal undamaged fibres of EX group. These results reinforce those from the 7th dpi, indicating that SB effectively delays the myotubes formation rate and their maturation process, and demonstrating the importance of muscle recruitment during SMR. The circularity results also corroborate this SB-induced effect, since SED group have now presented significantly higher values compared to EX group ($p<0.01$), suggesting that SED fibres maintained a more typical morphology of regenerating fibres (Schmalbruch, 1990), i.e., a more rounded shape, contrary to EX fibres whose morphology was similar to those of fully developed fibres, i.e., a more polygonal morphology (Wang, et al., 2014) with of decreased circularity values. Additionally, regarding the FT area, SED group not only showed, again, augmented FT accretion compared to EX group ($p<0.01$), but also remained with increased FT area when compared to Sham muscles ($p<0.01$). These results confirmed those from the 7th dpi, supporting that SB

induces fibroblasts to both increase and extend the FT production, promoting a more unfavourable skeletal muscle phenotype.

Conclusively, considering data from both the 3rd and the 7th dpi – the increased susceptibility to damage and the decreased myotubes CSA of EX groups, respectively – one could conceive that SB has beneficial effects during SMR, however, our data clearly shows that increased muscle recruitment, through voluntary running, has important effects on both myoblasts and fibroblast functionality during SMR. Indeed, the importance of mechanical tension in governing stem cells fate has already been addressed (Engler, et al., 2006), indicating that these cells essentially need mechanical signs to improve their functionality.

CONCLUSION

The presented data shows that SB induces major detrimental effects during SMR, delaying its time course and quality after CTX-induced injury. SB impairs myotubes functionality, i.e., decreases their formation rate and muscle mass growth, and increases FT deposition. Conversely, the increased muscle recruitment promoted by voluntary running during SMR seems pivotal to promote a faster and greater myotubes formation rate resulting in an earlier replacement of the injured skeletal muscle fibres by new ones and not by fibrotic tissue, resulting in the development of a more favourable skeletal muscle phenotype.

Ultimately, considering that skeletal muscle fibre replenishment by fibrosis represents the worst post-injury phenotypic adaptation and negatively affects the tissue quality and functionality, our results suggest that SB should be

avoided and moderate muscular recruitment should be performed during SMR.

Table 1 – Muscle damage semi-quantitative data in sedentary (SED) and exercise (EX) groups induced by cardiotoxin on the 1st, 3rd, 7th and 15th day post-injection (dpi). Total damage value is the sum of scores (ranging from 0 to 3 values in ascending order of histological alterations compared to the normal structure) of the cellular degeneration; inflammatory activity; necrosis extent and tissue disorganization.

Dpi	Groups	Cellular degeneration	Inflammatory activity	Necrosis	Tissue disorganization	Total damage
1	SED	3 (3-3)	3 (3-3)	3 (2-3)	3 (3-3)	12 (11-12)
	EX	3 (3-3)	3 (3-3)	3 (2.8-3)	3 (3-3)	12 (11-12)
3	SED	2 (1-3)[a]	3 (3-3)	1 (0.8-1)[a]	1 (1-2)[a]	7 (6-9)[a]
	EX	3 (2-3)	3 (3-3)	2 (2-2.8)	2 (2-3)	10 (9.3-11.8)
7	SED	1 (0-1)[a]	3 (2-3)	0 (0-0)[a]	1 (0-1)[a]	4 (2-5)
	EX	0 (0-0)	3 (3-3)	0 (0-0)	0 (0-0)	3 (3-3)
15	SED	0 (0-0)	0.5 (0-1)	0 (0-0)	0 (0-0)	0.5 (0-1)
	EX	0 (0-0)	0 (0-1)	0 (0-0)	0 (0-0)	0 (0-1)

Values are given as median (IQR: 1st – 3rd quartile). [a]$p<0.05$ versus EX CTX muscle within each dpi.

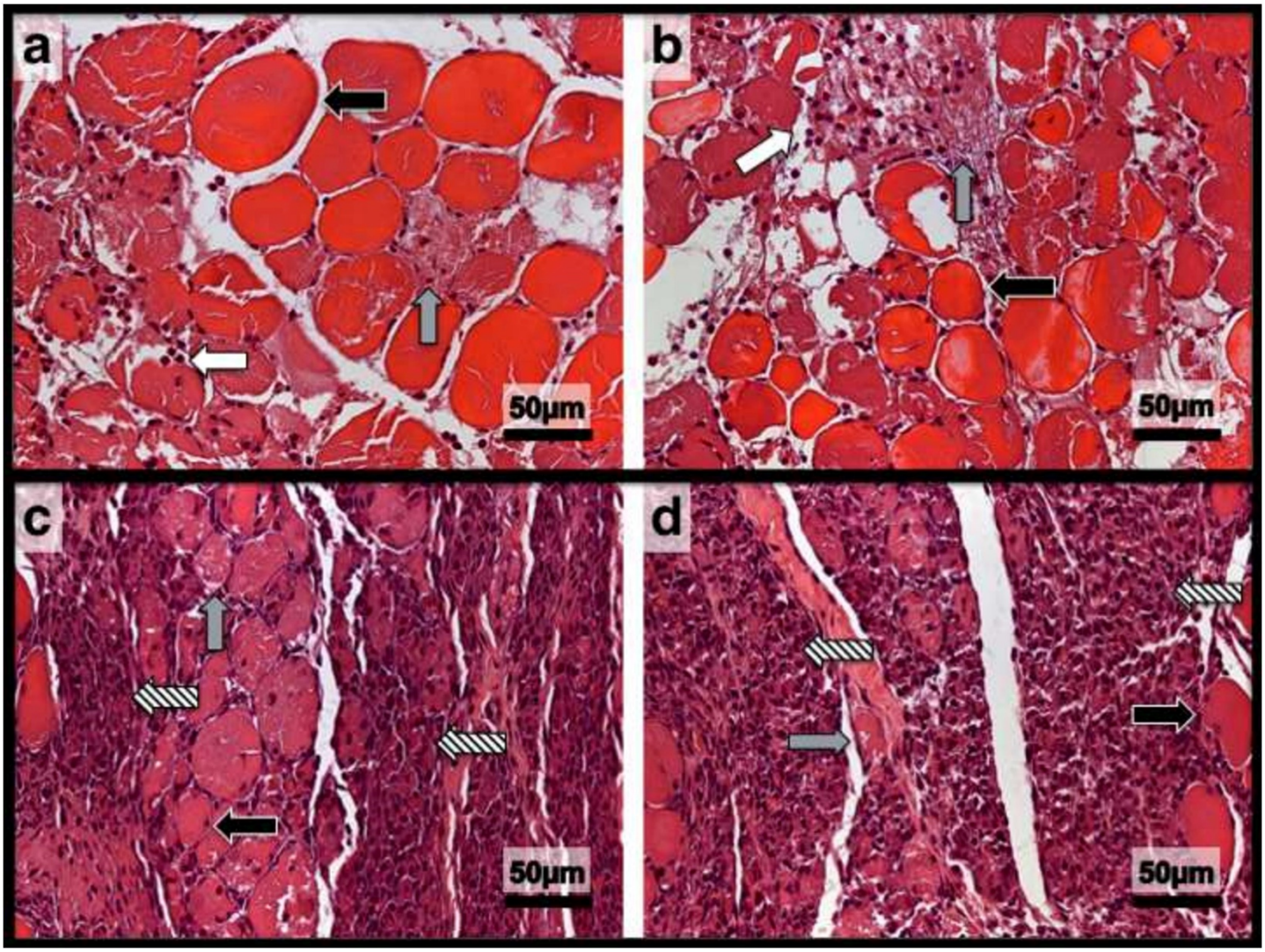
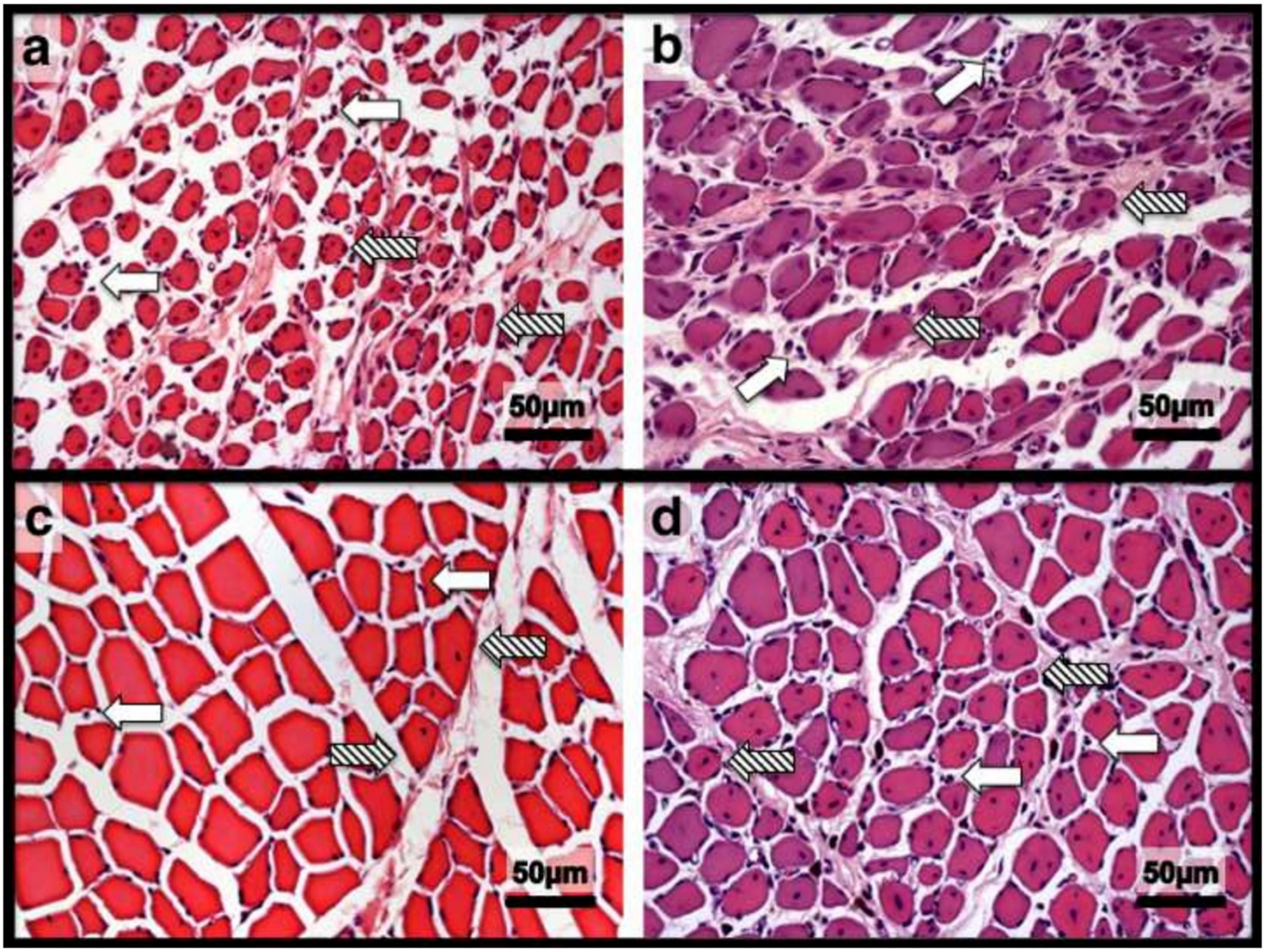


Figure 1 – Representative images of the tibial anterior muscles, stained with H&E, from exercise (**a** and **c**) and sedentary (**b** and **d**) groups injected with cardiotoxin and sacrificed on 1st and 3rd day post-injection (**a**-**b** and **c**-**d,** respectively). On **a** and **b**, it is clear a vast inflammatory infiltrate (white arrows), the presence of many necrotic fibres which are characterised by fragmented and infiltrated sarcoplasm (grey arrows), and the presence of hypercontracted enlarged and rounded fibres with eosinophilic (bright red sarcoplasm) staining (black arrows). On **c** and **d**, most of the area is covered with many small fibres, i.e., myotubes with central nuclei with basophilic (slightly purple) staining, possibly due to large amounts of RNA, in these actively differentiating and growing cells (striped arrows).



Cell and Tissue Research

Figure 2 – Representative images of the tibial anterior muscles, stained with H&E, from exercise (**a** and **c**) and sedentary (**b** and **d**) groups injected with cardiotoxin and sacrificed on 7st and 15th day post-injection (**a-b** and **c-d,** respectively). On **a** and **b**, many small muscles fibres with central nuclei (myotubes) are observed in both groups (striped arrows). There are marked histological differences between groups, regarding the myotubes shape and the fibrotic tissue quantity. On **c** few fibres are still regenerating (striped arrows) compared to **d**. Moreover, a noticeable extracellular matrix clearance on **a** and **c** contrasts with that on **b** and **d** which suggests prolonged exudate through excessive inflammatory infiltrate (white arrows).

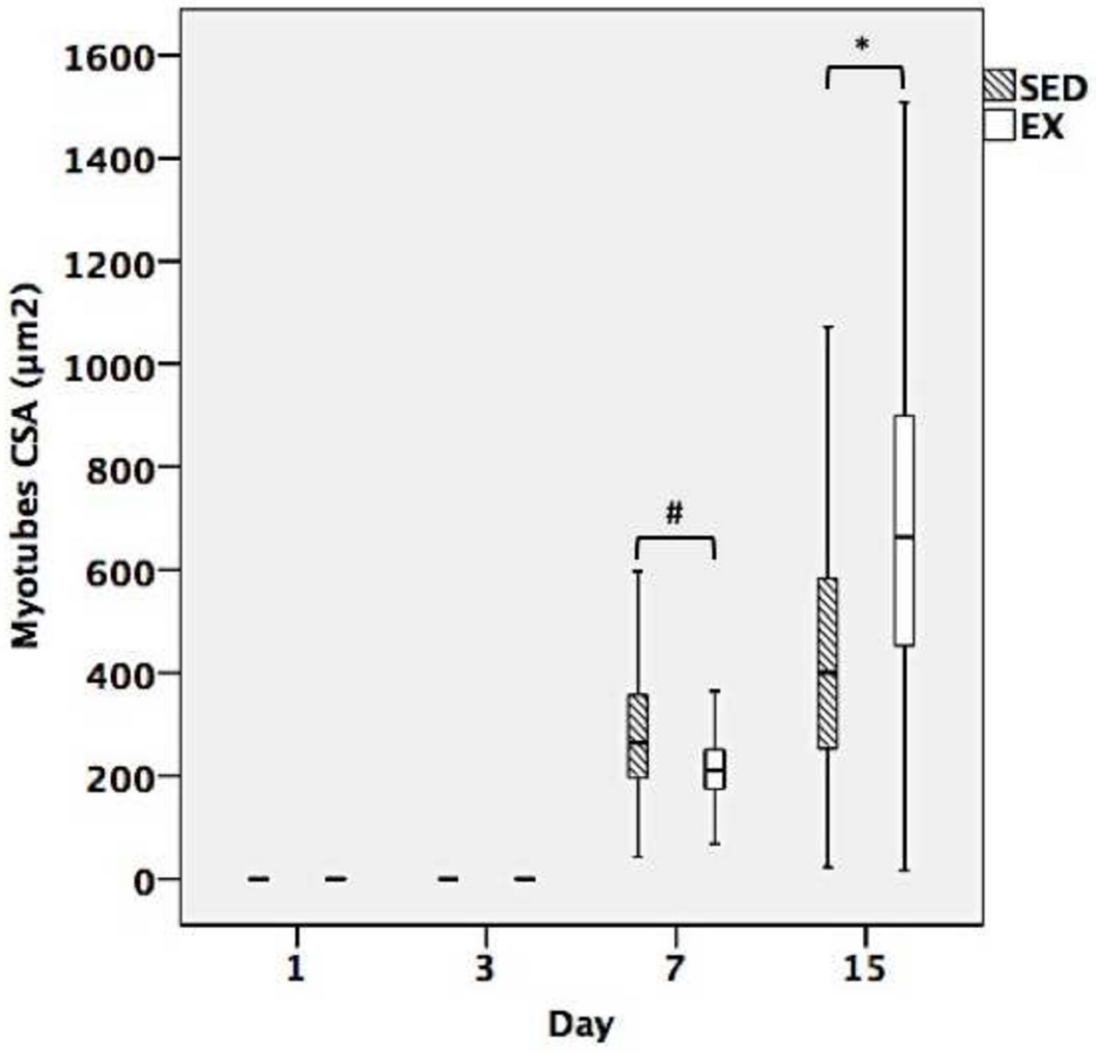


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Figure 3 – Distribution of myotubes cross sectional area (CSA) of muscles injected with cardiotoxin in each day post-injection, from sedentary (SED) and exercise (EX) groups. Box = median, 25 to 75%; T-bars = minimum and maximum values. # significantly higher than EX group ($p<0.01$). * significantly higher than SED group ($p<0.01$).

Figure 4

Click here to download Figure Fig4.tiff

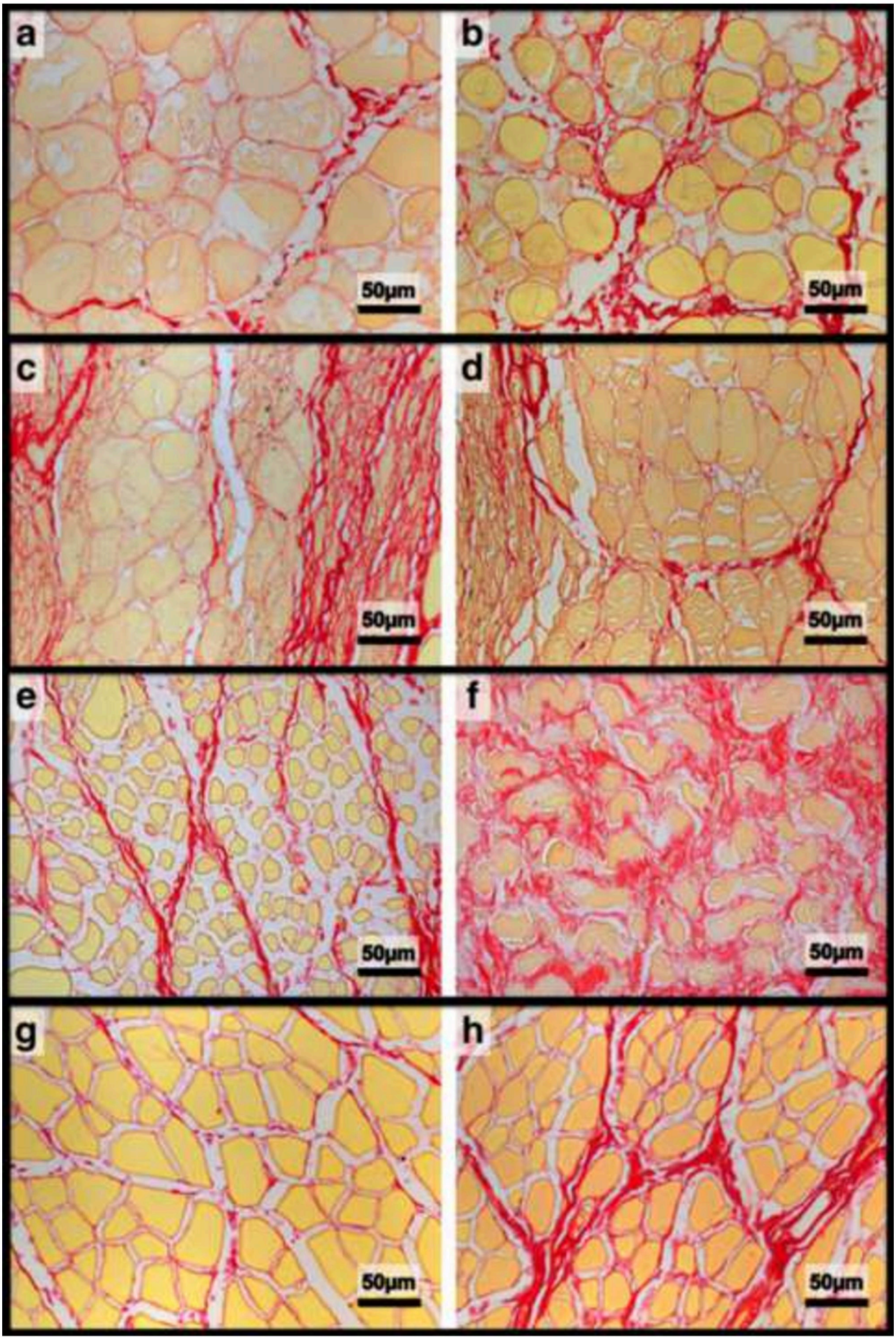
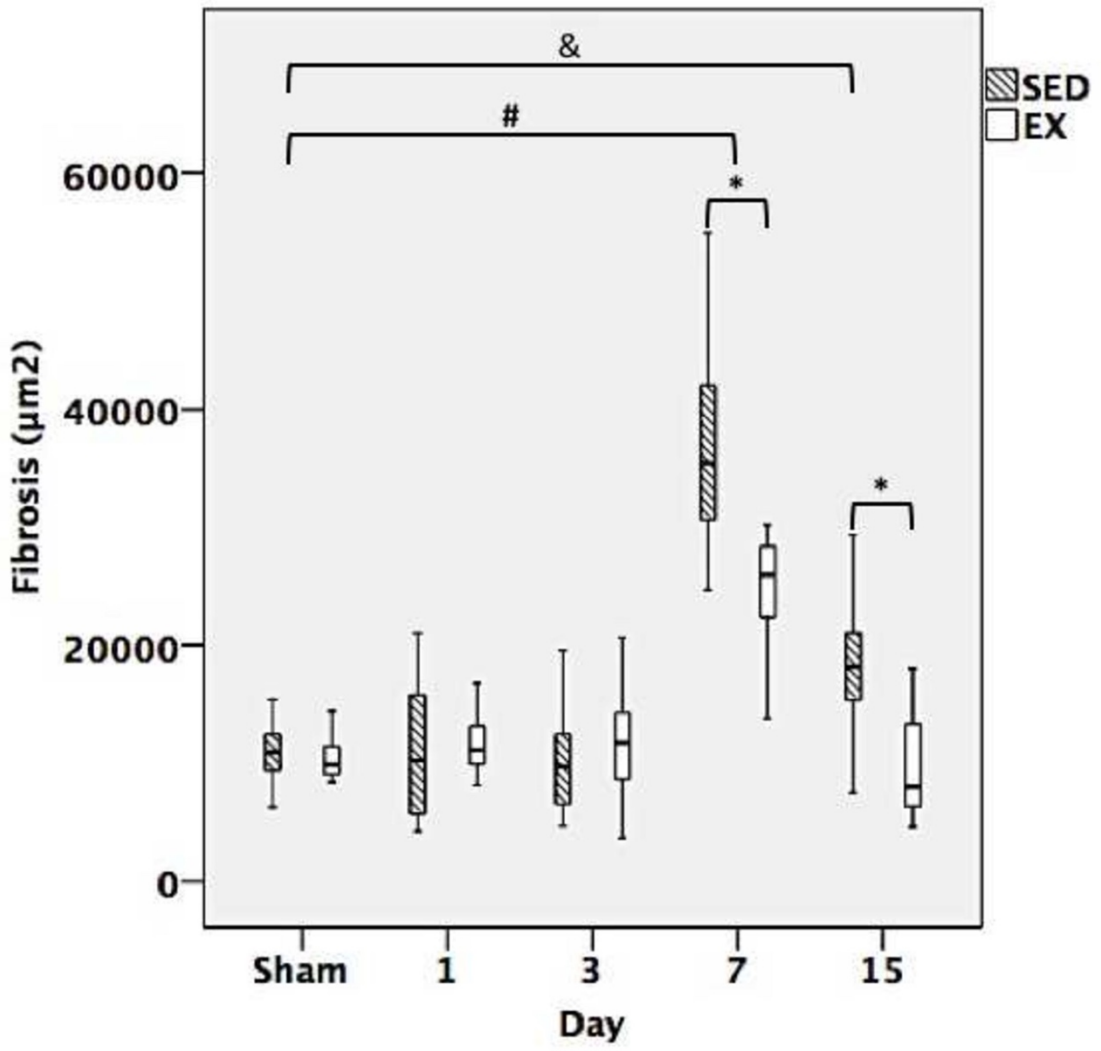


Figure 4 – Representative images of the tibial anterior muscles, stained with picrosirius red, from exercise (**a**, **c**, **e** and **g**) and sedentary (**b**, **d**, **f** and **h**) groups injected with cardiotoxin and sacrificed on the 1st, 3rd, 7st and 15th day post-injection (**a**-**b**, **c**-**d**, **e**-**f** and **g**-**h**, respectively).



Cell and Tissue Research

Figure 5 - Distribution of fibrotic tissue area of muscles injected with saline solution (Sham: overall median values of control muscles) and cardiotoxin in each day post-injection, from sedentary (SED) and exercise (EX) groups. Box = median, 25 to 75%; T-bars = minimum and maximum values. * significantly higher than EX group ($p<0.01$); # significantly higher than Sham muscles ($p<0.01$); & SED group significantly higher than Sham muscles ($p<0.01$).

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

All applicable institutional guidelines for the care and use of animals were followed. All procedures performed in this study involving animals were in accordance with the ethical standards of the institution.

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[Study 2]

Levels of physical activity modulate the tissue inflammatory response and the healing process in rat skeletal muscle

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Under Review

Levels of physical activity modulate the tissue inflammatory response and the healing process in rat skeletal muscle

Running heading: Sedentary behaviour delays muscle regeneration and promotes scar tissue formation during muscle healing.

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Abstract

This study tested the hypothesis that sedentary behaviour (SB) modulates the tissue inflammatory response and skeletal muscle repair (SMR) in male *Wistar* rats after cardiotoxin (CTX)-induced injury. Singly caged rats spent 8 weeks either as a sedentary group (SED, n=15) – in cages without running wheel – or as a control group (EX, n=15) – in cages with running wheel for voluntary running. Subsequently, all rats had each tibial anterior muscles infused, either with CTX (CTX; right muscle) or saline solution (Sham; left muscle), and were sacrificed (n=5 per group) on the 1st, 7th and 15th day post-injection (dpi). Histological and immunohistochemical analyses from the muscle damaged area

were used for calculating the myotubes percentage and the fibrosis accretion, and for measuring the number of M1 and M2 macrophages subtypes, and neutrophils. SED group showed a clearly exacerbated pro-inflammatory response, presenting increased numbers of both neutrophils (in all assessed days) and M1 macrophages (7^{th} and 15^{th} dpi) compared to EX group ($p<0.01$). EX group showed increased number of M2 macrophages on the 1^{st} dpi. On the 7^{th} dpi, SED group showed lower myotubes percentage compared to EX group ($p<0.01$) and, on the 15^{th} dpi, showed only 54% of normal undamaged fibres compared to 90% from EX group ($p<0.01$). SED group showed increased fibrosis on both 7^{th} and 15^{th} dpi. Our results show that SB affects the inflammatory response, enhancing and prolonging the Th1 phase, and delays and impairs the SMR process, as indicated by the number of myotubes and the fibrosis accretion.

Keywords: injury, regeneration, fibrosis, histology, repair

Key points: 1 – Levels of physical activity influence the muscle inflammatory response upon injury; 2 – Sedentary behaviour (SB) exacerbates the pro-inflammatory cellular response during muscle repair; 2 – SB delays muscle regeneration and promotes scar tissue formation during muscle healing.

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Conflict of Interest: Eduardo Teixeira, Juliana Garcia, Júlio Pacheco, and José Duarte declare that they have no conflict of interest.

1. Introduction

The inflammatory response to skeletal muscle injuries comprises intricate cellular and molecular interactions between both the skeletal muscle and the inflammatory cells [1]. Generally, upon muscle injury, the inflammatory response is characterized by a pro-inflammatory (or Th1) phase, mostly performed by neutrophils and pro-inflammatory macrophages (M1) that, despite causing further tissue damage, effectively stimulate the satellite cells (SCs) activation and proliferation stages. This Th1 phase is followed by an anti-inflammatory (or Th2) phase, performed by anti-inflammatory macrophages (M2), that reduces the Th1 phase by promoting inflammatory resolution, and stimulate SCs terminal differentiation and myoblasts growth and maturation, endorsing tissue repair [1, 2]. This important complex cellular relationship influences the skeletal muscle repair (SMR) by modulating the function of the myogenic stem cell population, which is fundamental to cope appropriately with different insults and to promote successful muscle repair [3]. In fact, both the importance of the neutrophils contribution to a successful muscle regenerative response [4, 5] and the negative impact of an impaired macrophage polarization during SMR [6] have already been addressed.

Besides other factors influencing SMR (e.g., age, genetics, and insult severity and nature) it is known that different skeletal muscle loading conditions also modulates its regenerative capacity. Indeed, increased muscle recruitment, through increased physical activity or exercise training, appears to be beneficial in improving SCs functionality, and impairing fibrosis during SMR [7]. Moreover, some indirect evidences obtained in different settings showed that acute muscle recruitment seems to alter macrophages functions, e.g., increasing their phagocytic activity [8], as well as their tumor cytotoxicity and chemotaxis capacities in both acute and chronic loading conditions [9]. Nevertheless,

studies addressing the effects of different levels of muscle recruitment, through sedentary behaviour or increased voluntary physical activity, on the SMR process, are still scarce. Currently, this specific matter has been widely addressed because sedentary behaviour (SB) is considered a risk factor for multiple organs dysfunction, particularly for skeletal muscle [10]. Indeed, this condition has profound negative effects on the skeletal muscle phenotype, affecting its morphology, composition and function [11], and probably its regenerative capacity; nevertheless, the effects of sedentary behaviour on SMR cellular dynamics, i.e., SCs, fibroblasts, and immune cells functions and their interaction are unknown. Therefore, the aim of this study was to test the hypothesis that sedentary behaviour impairs SMR, determining if 8 weeks of decreased muscle recruitment alters the local inflammatory response and the healing process, on rat tibial anterior muscle in response to a cardiotoxin (CTX)-induced damage.

2. Material and methods

2.1. Animals and experimental design

After arrival, thirty male *Wistar* rats with 2 months old (Charles River Laboratories, Barcelona, Spain) were individually caged and kept in a controlled environment (constant humidity of 50-60% and temperature of 22±2 °C) and in an inverted 12h light/dark cycle. Standard rat chow and tape water were provided *ad libitum* during the entire protocol. Following one week of quarantine, rats were randomly assigned to either a sedentary group (SED, n=15) or an exercise control group (EX, n=15). EX rats, housed individually in cages equipped with a complete activity wheel system (Tecniplast 2154F0105; overall dimensions of 480x315x470 mm^3) were able to run voluntarily. SED rats, also housed individually in similar cages but without the activity wheel system, were forced to sedentary behaviour. After 8 weeks in these conditions each animal, under light

anaesthesia induced by an intraperitoneally (ip) injection of ketamine and xylazine, had his right tibial anterior muscle injected with 30 µl of 20 µM cardiotoxin (CTX; Naja mossambica mossambica; C9759, Sigma-Aldrich) in the mid belly portion (CTX muscle) and his left tibial anterior muscle injected with 30 µl of saline solution (Sham muscle) in the same muscle region. Lyophilized CTX venom was prepared by liquefying a recently opened container in PBS. The fresh solution was divided into Eppendorf tubes as 10 ml aliquot stocks, flash frozen, and stored at -80 ºC. Each tube was thawed fresh before injection and not re-used. Injections where executed using a 50 µl, 22-gauge needle-fixed syringe (Hamilton; 20736, Sigma-Aldrich) and to preserve the experimental procedure consistency, all the injections were made by the same researcher, certifying that similar infiltrations were done to all groups. Subsequently, all animals were kept in the same conditions and 5 animals from SED and EX groups were sacrificed on the 1st, 7th and 15th day post-injury (dpi). Total distance ran by each rat of EX group was documented daily. The study was approved by the ethics committee of the Faculty of Sport from the University of Porto.

2.2. Tissue collection and processing for light microscopy

All animals were sacrificed by a deadly ip injection of ketamine and xylazine and the tibial anterior muscles removed, cleansed of contiguous soft and fat tissue, cut in half in the mid belly region and fixated in a solution containing 4% paraformaldehyde (P6148; Sigma-Aldrich), 2,5% sucrose (S0389; Sigma-Aldrich) and 0,1% glutaraldehyde (G5882; Sigma-Aldrich) in PBS (pH 7.2) at 4ºC for 24 hours. After fixation, both halves were dehydrated through graded ethanol solutions, cleared in xylene and embedded in paraffin blocks. Transverse 5 µm thick sections from each muscle part were cut on a Leica 2125 rotary microtome (Leica Microsystems). The created slides were then used for muscle

histomorphometry, evaluation of fibrotic tissue accretion, and for immunohistochemistry analysis.

2.3. Histomorphometry, fibrosis and immunohistochemistry analysis

For histomorphometry, muscle sections were stained with haematoxylin and eosin (H&E), examined in a light microscope (Axio Imager A1, Carl Zeiss; Germany) with a 40x magnification objective and images snapped by an attached digital camera with Axio Vision 4.7 software. Obtained non-overlapping images were then analysed with ImageJ software (NIH, Bethesda, MD) to quantify the morphological features of the muscles sections. For consistency, the histomorphological evaluation was accomplished on multiple single frame images (5 to 10 images obtained per section) acquired from the same muscle section (9 different sections analysed per muscle) and, exclusively, within the damaged area, i.e., muscle area with clear inflammatory infiltrate, presence of necrotic fibres (degenerating fibres with fragmented sarcoplasm), regenerating fibres (small basophilic and central nucleated fibres with normal sarcoplasm) or excessive fibrosis. The level of CTX-induced muscle damage was assessed by calculating the percentage of necrotic fibres from the total number of analysed cells in each image during the 1st dpi. The SMR progress was evaluated by counting the percentage of myotubes (small basophilic and central nucleated fibres with normal sarcoplasm) and the fibrotic tissue accretion at each dpi. Percentage of myotubes and normal fibres, i.e., fibres with peripheral nuclei, intact sarcolemma and non-fragmented sarcoplasm, was determined from the total number of analysed cells in each image.

For fibrosis evaluation we applied the picrosirius red (SR) staining method [12]. Briefly, slides were incubated on 0,1% SR in saturated picric acid for 1h, rinsed in 0,5 % acetic acid, dehydrated in ethanol and cleared in xylene. This technique stains collagen tissue

bright red and muscle tissue yellow. Again, 5 to 10 non-overlapping single frame images, acquired from the same muscle section and within the damaged area were examined with Image-Pro Plus 6.0 software (Media Cybernetics). The area covered by fibrosis was quantified in all experimental groups.

The immunohistochemistry analysis was performed, as described by others [13], to quantify M1 and M2 macrophages subtypes, and neutrophils present in the muscle damaged area. Briefly, slides were deparaffinized and put in a pressure cooker for 20 min in 10 mM citrate buffer, pH 6.0, for the antigen retrieval procedure. After cooling and washing twice with PBS solution for 5 min, the endogenous peroxidase activity was blocked, during 30 min, with a fresh 3% solution of hydrogen peroxide in methanol. The non-specific binding sites were blocked with 3% bovine serum albumin (BSA) for 30 min. Following the blocking step, each slide was incubated with antibodies targeted to M1 and M2 macrophages subtypes, and neutrophils, respectively, anti-CD68 antibody (ab125212; abcam), anti-mannose receptor antibody (ab64693; abcam), and anti-neutrophil elastase antibody (ab 21595; abcam), all diluted (1:100) in PBS-T overnight (4ºC). Sections were washed with PBS and then probed with goat anti-rabbit IgG horse radish peroxidase secondary antibody (1:200) (ab97051; abcam) in PBS-T for 2 h at 37 ºC. The sections were then washed twice, under gentle stirring with PBS for 5 min, and incubated with 3,3′-diaminobenzidine tetrahydrochloride (DAB) reagent (D0426; Sigma-Aldrich) for 3 min. After washing, the slides were then counterstained with a solution of hematoxylin diluted in water (1:15) for 3 min. As a negative control, additional sections were treated similarly, however the primary or secondary antibodies were substituted by PBS. Finally, slides were mounted and cover slipped and analysed by light microscopy as already described. Additionally, since anti-CD68 antibody also reacted with early developing myotubes, their total number within the analysed area was also measured.

This procedure, together with the classic evaluation of central nucleated fibres with H&E staining, allow to distinguish different phases of myotubes evolution.

3. Statistical analyses

To determine within-group normality for a given variable the Kolmogorov-Smirnov test was performed and the Levene´s test to determine homogeneity of variance. Normal distributed variables are presented as mean and standard deviation. However, considering that most variables lacked normal distribution, the differences between groups and the differences on each dpi within groups were tested with the Mann-Whithney (two-tailed) test and the Kruskal-Wallis test, respectively. These variables are presented as median and interquartile range (IQR: 1st – 3rd quartiles). SPSS, version 21, was used to perform all statistical analyses. Statistical significance was accepted at $p<0.05$.

4. Results

4.1. Body weight and voluntary physical activity

SED body weight was significantly higher than EX (417.6±35.5 g vs. 384.4±26.8 g, respectively, $p<0.01$). During the 8 weeks, EX group ran on average 19 ± 12.4 km/week.

4.2. Hystomorphometry, fibrosis and immunohistochemistry analysis

As illustrated in figure 1, injections with CTX produced evident skeletal muscle damage in both groups when compared to Sham muscles, which showed very restricted areas of muscle damage, possibly produced by the needle mechanical injury. To accurately examine the progression of the inflammatory cells infiltration, and the development of fibrotic tissue accretion throughout the experimental period in CTX muscles, comparisons were made with data obtained from Sham muscles. Images illustrating tibial

anterior muscles sections, stained with SR, observed in all groups, and related fibrosis area distribution, are depicted in figure 2. Representative images of immunohistochemistry from M1 and M2 macrophages, and Neutrophils in the tibial anterior muscles sections, and corresponding quantitative analyses, are illustrated in figure 3, 4 and 5, respectively.

1st day post-injury

In the infused region, EX and SED groups showed similar percentages of necrotic fibres (87% vs. 89%, respectively; $p>0.05$), no presence of myotubes, as expected (figure 1), and, as depicted in figure 2, similar quantity of fibrotic tissue area [11.7 (5.9 – 15.9) mm^2 and 11.1 (9.3 – 13.8) mm^2 for SED and EX groups, respectively, $p>0.05$]. Both EX and SED groups showed similar number of M1 macrophages, as illustrated in figure 3 ($p>0.05$). SED group showed decreased number of M2 macrophages (figure 4) compared to EX group ($p<0.01$). Finally, SED group showed increased number of neutrophils (figure 5) compared to EX group ($p<0.01$).

7^{th} day post-injury

SED group presented a decreased percentage of myotubes, compared to EX group (58,4% vs. 69,3%, respectively, $p<0.01$), and, as depicted in figure 2, increased fibrosis [36.1 (34.1 – 43.4) mm^2 vs. 24.9 (22.3 – 28.3) mm^2 for SED and EX groups, respectively, $p<0.01$]. SED group showed increased number of M1 macrophages (figure 3) compared to EX group ($p<0.01$). Additionally, SED group showed increased number of developing myotubes, detected by immunohistochemistry as illustrated in figure 3, compared to EX group [5.5 (2 – 15.8) vs. 0 (0 – 1.8), respectively, $p<0.01$]. Both EX and SED groups showed similar number of M2 macrophages, as depicted in figure 4 ($p>0.05$). Finally,

SED group showed increased number of neutrophils (figure 5) compared to EX group ($p<0.01$).

15th day post-injury

SED group presented an increased percentage of myotubes, compared to EX group (46% vs. 10.2%, respectively, $p<0.01$). Again, as illustrated in figure 2, SED group showed increased fibrosis [17.9 (15.4 – 21.3) mm^2 vs. 8.4 (6 – 14) mm^2, respectively, $p<0.01$]. SED group showed increased number of M1 and M2 macrophages, and neutrophils (as illustrated in figures 3, 4, and 5, respectively), compared to EX group ($p<0.01$).

Briefly, as depicted in figure 2, both SED and EX groups showed increased area of fibrosis throughout the experimental period, and increased number of M2 macrophages (figure 4), compared to Sham muscles ($p<0.01$). Regarding the number of M1 macrophages (figure 3) and neutrophils (figure 5) on the 1st and 7th dpi, both SED and EX groups presented increased numbers of these inflammatory cells ($p<0.01$), however, only SED group showed their numbers augmented on the 15th dpi, compared to Sham muscles ($p<0.01$).

5. Discussion

Our results demonstrate that sedentary behaviour (1) deeply affects the local inflammatory cellular response upon injury, enhancing and prolonging the Th1 phase, and (2) delays/impairs the SMR process, as indicated by the number of myotubes and the fibrosis accretion throughout the experimental period.

On the 1st dpi, CTX-induced damage was similar between SED and EX groups, who presented no differences in the percentage of necrotic fibres. These results demonstrate

that the injection protocol was homogenous between groups and that different patterns of muscle recruitment before the damage protocol did not changed the vulnerability to CTX-related effects. Despite similar number of M1 macrophages, SED group showed increased number of neutrophils compared to EX group, suggesting that SB may prone inflammatory cells to rapidly endorse the Th1 response. Data from M2 macrophages seem to corroborate this hypothesis. Interestingly, considering the importance of the tissue microenvironment and the extrinsic factors, e.g., the surrounding cytokines, in governing macrophages polarization [14], it is appealing to consider that increased muscle recruitment before and during the SMR, acutely stimulates macrophages to polarize into an anti-inflammatory state. In fact, EX group showed increased number of M2 macrophages, compared to SED group, only 24h post-injury.

On the 7th dpi, SED group developed an apparent unfavourable response, showing lower myotubes percentage, increased number of developing myotubes (indicative of a delayed evolution), and increased fibrosis, compared to EX group, suggesting an impaired muscle regeneration and an excessive scar tissue formation. Recently, the positive effects of increased muscle loading, through extracorporeal shock wave, during SMR – enhancing SCs number, their proliferation and differentiation rates – on rat skeletal muscle have been documented [15], and some human studies also showed that increasing muscle tensions, through eccentric exercise training, has clear effects on the improvement of the skeletal muscle to a favourable and more functional phenotype during SMR [16, 17]. Our results suggest that decreasing muscle recruitment during SMR delays and impairs the SCs ability to cope with injury when compared to the faster and greater myotubes formation rate promoted by regular voluntary running. Considering that cluster differentiation (CD) 68 is also expressed by early developing cells allowing them to attach to selectins during maturation and, besides the M1 macrophage identification by CD68,

this immunohistochemistry technique also allowed the categorization of an earlier stage of myotubes development. In fact, the increased number of developing myotubes in SED group suggests a delayed myotube maturation when compared to EX group that presented increased number of myotubes without expressing CD68. Disregarding the lack of studies analysing the effects of increased muscle recruitment on the extracellular matrix dynamics, it is known that both increased voluntary running and exercise influence the collagen turnover and the matrix metalloproteinases (proteases that mainly degrade collagen) activity [18]. Moreover, others also showed that increased muscle loading successfully decreased fibrosis during SMR [19]. Despite the number of M2 macrophage did not differ between groups, SED group showed increased numbers of both M1 macrophages and neutrophils, i.e., representative inflammatory cells of the Th1 phase. Indeed, over the past years, both the pro-inflammatory environment promoted by sedentary behaviour [11] and the anti-inflammatory effects of regular physical activity have been acknowledged in several conditions [20], corroborating our results since sedentary behaviour exacerbated the Th1 phase, increasing the number of pro-inflammatory cells infiltrating the damaged tissue. Additionally, recent data evincing the effects of pro-inflammatory macrophages in both exacerbating tissue damage and increasing fibrosis has been addressed [21]. Thus, is seems appealing to consider that the aggravated Th1 inflammatory response induced by SB significantly impairs both the myotubes formation and the fibrotic tissue accretion. Moreover, considering also the emerging data linking the effects of anti-inflammatory macrophages in both decreasing fibrotic activity and boosting the resolution phase during tissue repair [21], our results suggest that the increased muscle recruitment enhance the Th2 inflammatory response at an early phase of the SMR, since EX group presented increased number of M2 macrophages on the 1st dpi. In fact, the anti-inflammatory effects of exercise during

musculoskeletal disorders has been recently proposed as a mean of therapeutic approach [22]. Furthermore, our results show that this anti-inflammatory effect of muscle recruitment decreases the number of the pro-inflammatory cells needed during SMR, accelerate the mytoubes maturation and decreases the fibrotic tissue accretion.

On the 15th dpi, SED group presented increased myotubes percentage compared to EX group, i.e., SED group showed only 54% of normal undamaged fibres compared to 90% from EX group. These results strengthen those from the 7th dpi, demonstrating that decreased muscle recruitment effectively delays the myotubes formation rate and their maturation process during SMR. Furthermore, SED group showed, again, increased fibrosis compared to EX group. These results support those from the 7th dpi, corroborating the detrimental effect inflicted by the reduced muscle recruitment in inducing the fibroblasts to excessively produce fibrotic tissue, culminating in the development of a more unfavourable skeletal muscle phenotype during SMR. SED group still showed increased number of all the inflammatory cells analysed, clearly demonstrating (1) an impaired tissue resolution, i.e., a prolonged accumulation of degenerated and/or necrotic cells and debris with the correspondent increase in the inflammatory infiltrate, and (2) an increased amount of time necessary to proper tissue healing, mainly achieved by scar tissue formation. Indeed, considering the proposed positive effects of both acute [8] and chronic [23] muscle recruitment on macrophages functions, our results suggest that SB may also impair this cell function, since SED group showed increased number of M1 macrophages, on both the 7th and the 15th dpi, demonstrating the need of additional macrophages to accomplish proper debris clearance. Considering data of the fibrosis overall progression, our results clearly show the regular scar tissue accretion during SMR. However, in normal physiological circumstances, this increased collagen deposition is usually reversible [24] and an unnecessary or impaired

scar tissue accumulation, like that showed by SED group, resembles that of pathological conditions [25]. Again, our data evince the negative effect of SB in the overall fibroblast response during SMR.

6. Conclusion

Our data clearly shows that SB, trough decreased muscle recruitment, has important detrimental effects during SMR, i.e., exacerbating the pro-inflammatory Th1 phase that results in an impaired myotubes development and fibroblast functionality. Additionally, our results also evince that increased muscle recruitment during SMR has important effects on macrophage response, apparently shortening the inflammatory response, and hindering an excessive accumulation of neutrophils. These results support our initial hypothesis that levels of physical activity modulate the tissue inflammatory response and the healing process, with SB negatively affecting the skeletal muscle ability to repair.

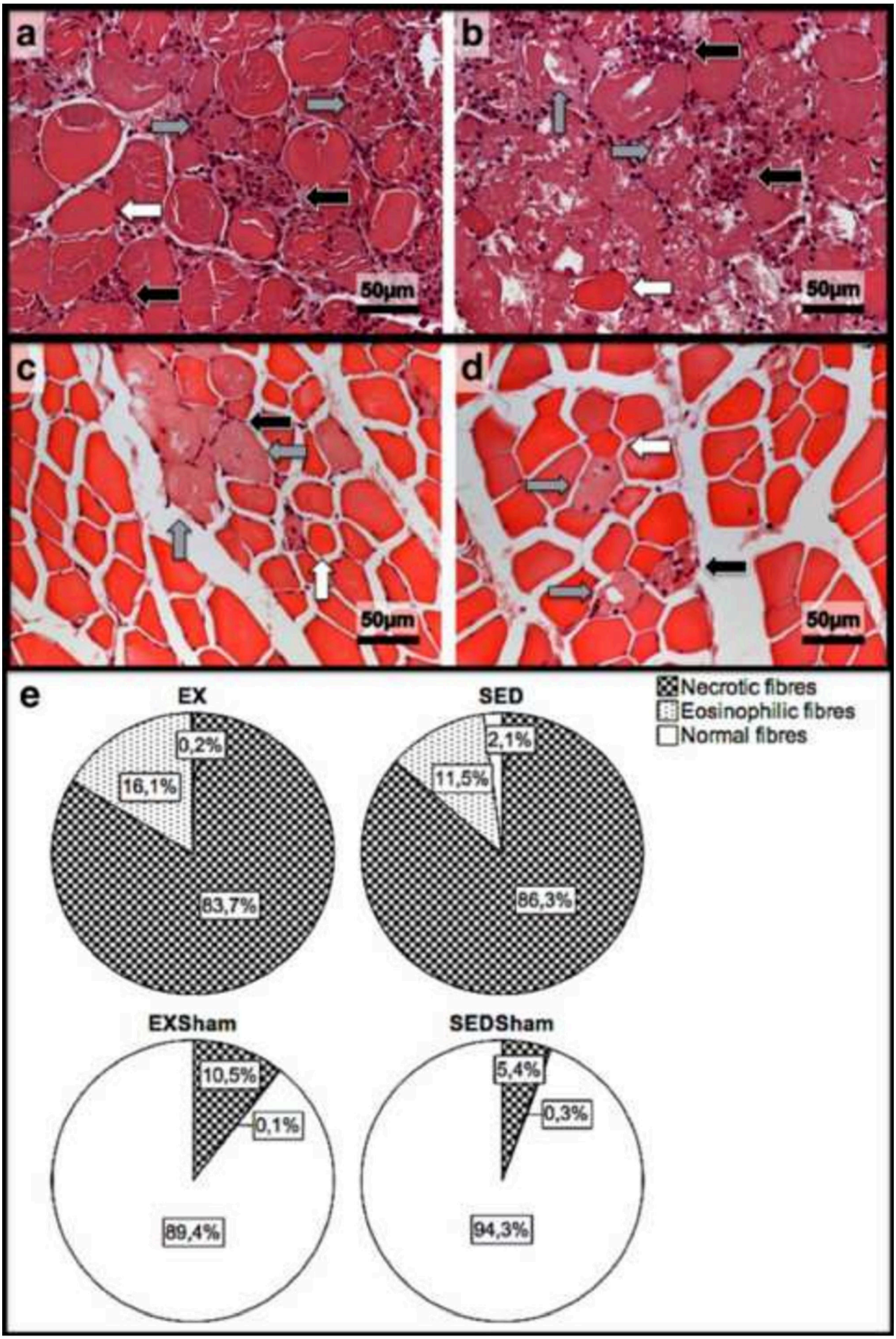

Fig. 1 – Representative images of the infused area of tibial anterior muscles stained with H&E, from exercise (EX) and sedentary (SED) groups injected with cardiotoxin (**a** and **b**), and saline solution (Sham; **c** and **d**) and sacrificed on the 1st day post-injection. In **e** the corresponding quantitative analyses of necrotic (fibres with fragmented and infiltrated sarcoplasm), eosinophilic (hypercontracted, enlarged, and rounded fibres with eosinophilic staining, i.e., bright red sarcoplasm), and normal (fibres with peripheral nuclei, intact sarcolemma and non-fragmented sarcoplasm) fibres is depicted. On **a** and **b**, it is clear a vast inflammatory infiltrate (black arrows), existence of several necrotic fibres (grey arrows), and existence of eosinophilic fibres (white arrows). On **c** and **d**, despite the presence of some necrotic fibres (grey arrows), inflammatory infiltrate (black arrows), and fewer eosinophilic fibres (white arrows), the majority are normal fibres. EX – Exercise group injected with cardiotoxin; SED – Sedentary group injected with cardiotoxin; EXSham – Exercise group injected with saline solution; SEDSham – Sedentary group injected with saline solution.

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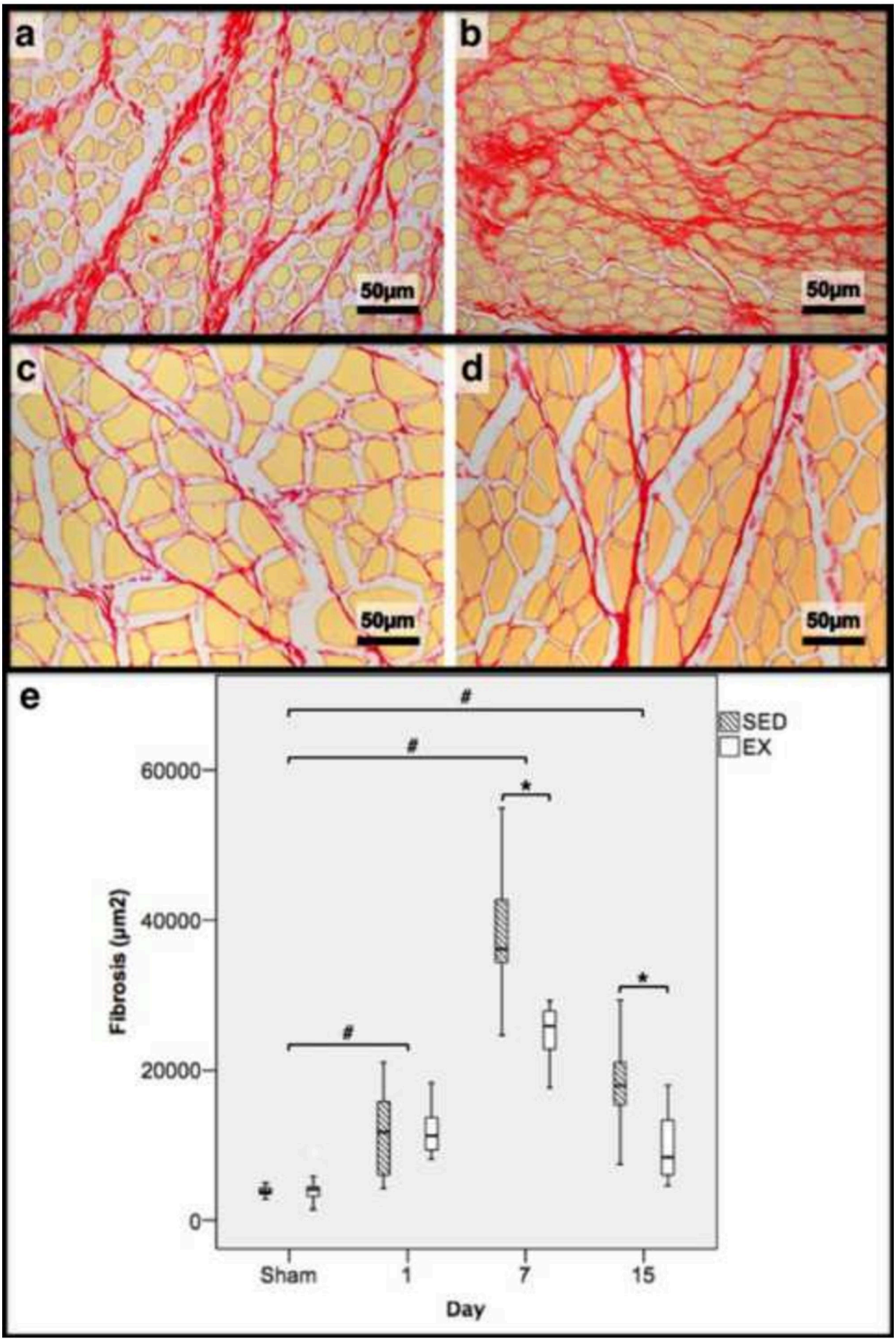

Fig. 2 – Representative images of the tibial anterior muscles, stained with picrosirius red, from exercise (EX; **a**, and **c**) and sedentary (SED; **b** and **d**) groups injected with and cardiotoxin, sacrificed on the 7th (**a** and **b**), and 15th (**c** and **d**) day post-injection. In **e** it is depicted the corresponding distribution of the fibrotic tissue area in all assessed days in both groups (Sham: overall median values of control muscles). Box = median, 25 to 75%; T-bars = minimum and maximum values. * significantly higher than EX group ($p<0.01$); # significantly higher than Sham muscles ($p<0.01$). SED – Sedentary group injected with cardiotoxin; EX – Exercise group injected with cardiotoxin; Sham – control muscles injected with saline solution.

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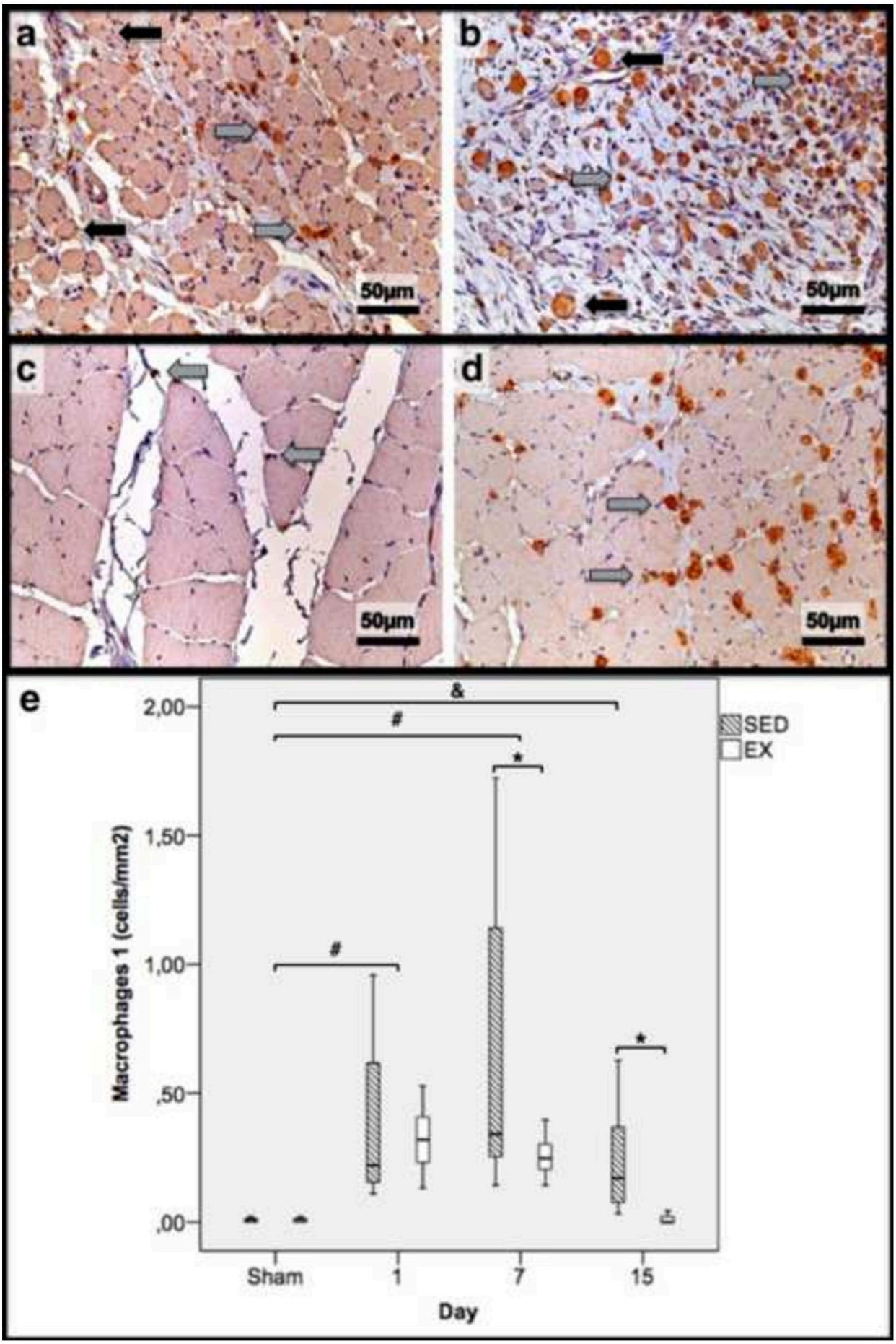

Fig. 3 – Immunohistochemistry of macrophages 1 (expressing CD68) in the tibial anterior muscles from exercise (EX: **a** and **c**) and sedentary (SED: **b** and **d**) groups injected with cardiotoxin, sacrificed on the 7th and 15th day post-injection (**a-b** and **c-d**, respectively), and corresponding quantitative analyses (**e**). Sham muscles in both EX and SED, injected with saline solution, represent the values obtained on the 1st day post-injection. The images clearly show the brown-stained M1 macrophages (grey arrows) infiltration, which is more pronounced in SED group (**b** and **d**). Additionally, it is also observed many small myotubes with central nuclei, mainly in **b**, expressing CD68 (black arrows). Box = median, 25 to 75%; T-bars = minimum and maximum values. ***** significantly higher than EX group ($p<0.01$); **#** significantly higher than Sham muscles ($p<0.01$); **&** SED group significantly higher than Sham muscles ($p<0.01$). SED – Sedentary group injected with cardiotoxin; EX – Exercise group injected with cardiotoxin; Sham – control muscles injected with saline solution.

Figure4_ppt

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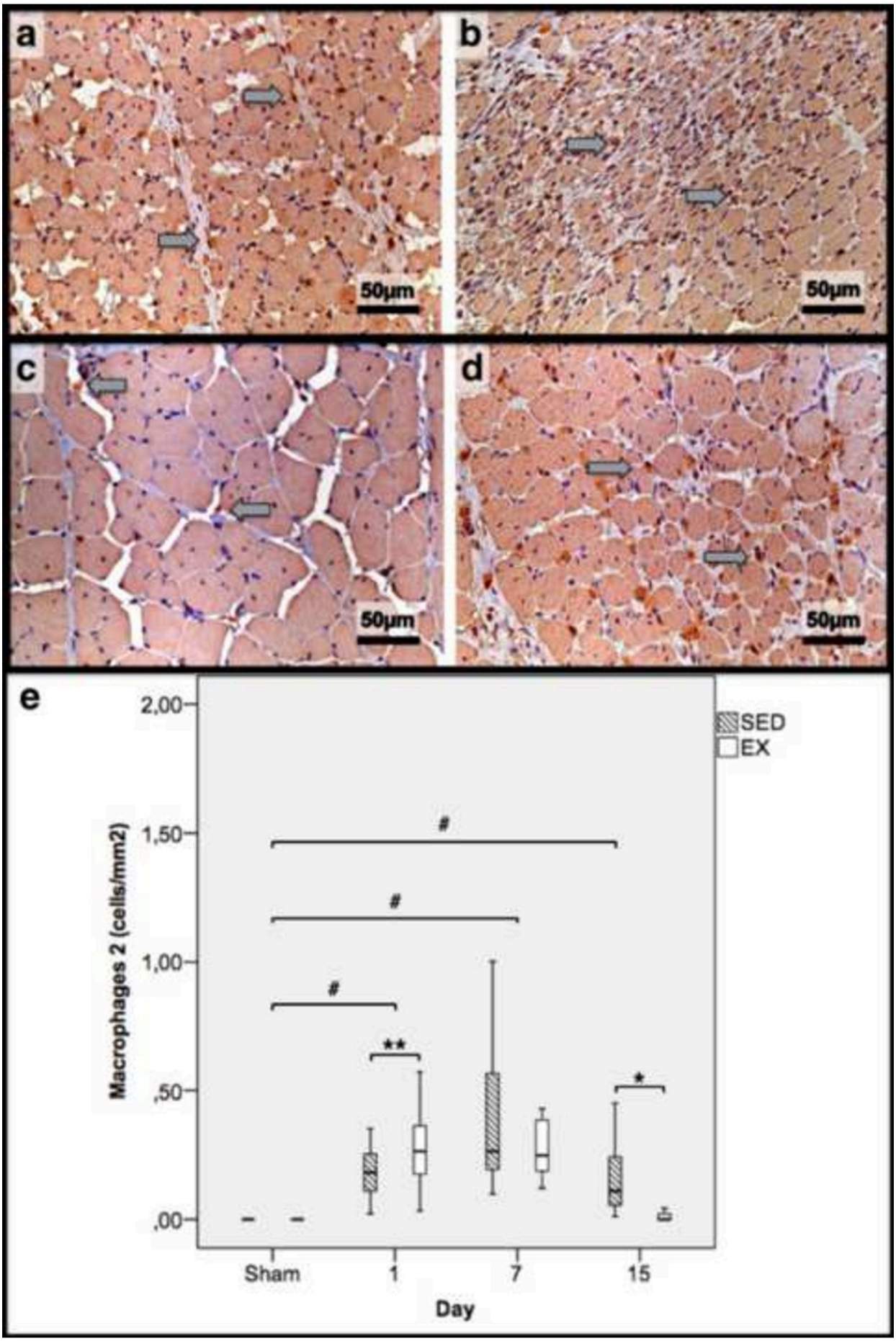

Fig. 4 – Immunohistochemistry of macrophages 2 (expressing mannose receptor) in the tibial anterior muscles from exercise (EX: **a** and **c**) and sedentary (SED: **b** and **d**) groups injected with cardiotoxin, sacrificed on the 7th and 15th day post-injection (**a-b** and **c-d**, respectively), and corresponding quantitative analyses (**e**). Sham muscles in both EX and SED, injected with saline solution, represent the overall values obtained on the 7th and 15th days post-injection. The images clearly show the brown-stained M2 macrophages (grey arrows) infiltration, which is more pronounced in SED group (**b** and **d**). Box = median, 25 to 75%; T-bars = minimum and maximum values. * significantly higher than EX group ($p<0.01$); ** significantly higher than SED group ($p<0.01$); # significantly higher than Sham muscles ($p<0.01$). SED – Sedentary group injected with cardiotoxin; EX – Exercise group injected with cardiotoxin; Sham – control muscles injected with saline solution.

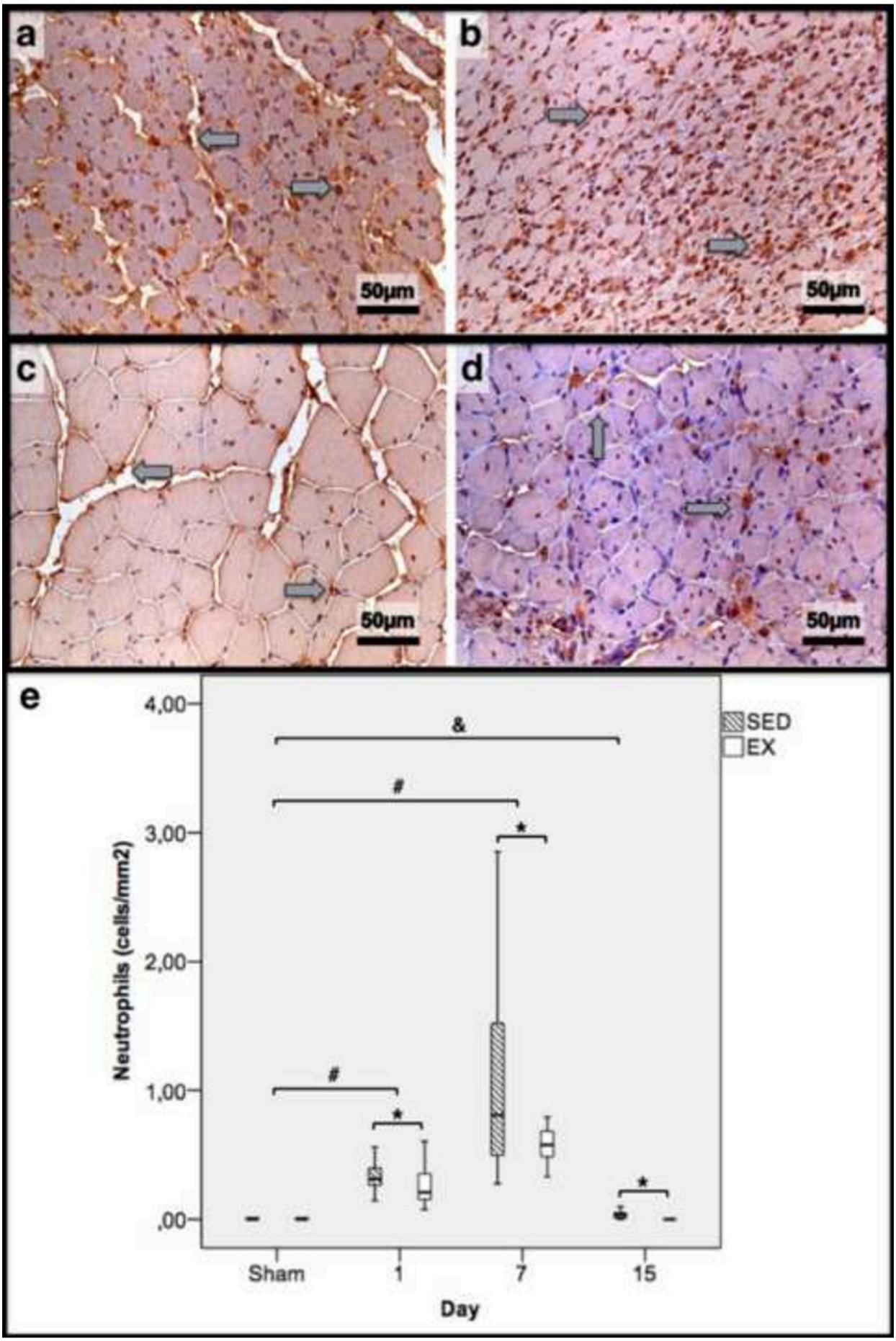

Fig. 5 – Immunohistochemistry of cells expressing neutrophil elastase in the tibial anterior muscles from exercise (EX: **a** and **c**) and sedentary (SED: **b** and **d**) groups injected with cardiotoxin, sacrificed on the 7th and 15th day post-injection (**a-b** and **c-d**, respectively), and corresponding quantitative analyses (**e**). Sham muscles in both EX and SED, injected with saline solution, represent the overall values obtained on the 1st day post-injection. The images clearly show the brown-stained neutrophils (grey arrows) infiltration, which is more pronounced in SED group (**b** and **d**). Box = median, 25 to 75%; T-bars = minimum and maximum values. * significantly higher than EX group ($p<0.01$); # significantly higher than Sham muscles ($p<0.01$). SED – Sedentary group injected with cardiotoxin; EX – Exercise group injected with cardiotoxin; Sham – control muscles injected with saline solution.

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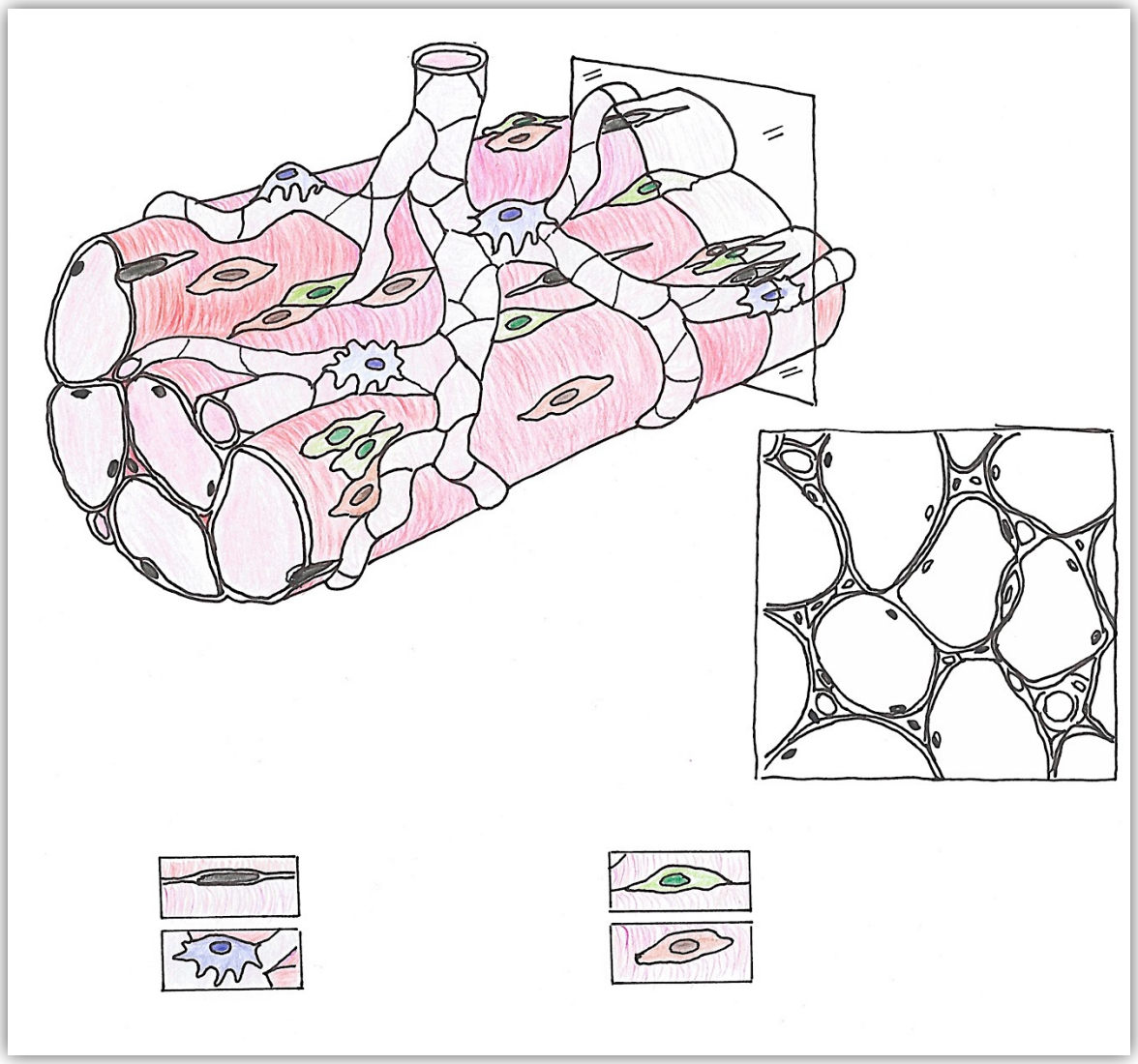
4. GENERAL DISCUSSION



This chapter comprises a discussion about the used methodology – addressing the reasons that substantiate the options for the used experimental protocol and the used technics to collect data – followed by an integral overview of the obtained results in the experimental studies.

Methodological Discussion

Considering the methodological difficulties and ethical limitations to appropriately investigate the influence of SB on the SMR process in humans, animal models are usually favoured to adequately address this important issue. A great variety of exercise models can be used to analyse the physiological effects of either strength (Lowe & Alway, 2002) or endurance training (Fonseca et al., 2011; Holloszy & Booth, 1976) in the rodents' skeletal muscle morphologic and biochemical characteristics. The most commonly used are the forced treadmill running, and the voluntary wheel running. Both models have their own specificities, advantages, and disadvantages. The use of forced exercise assures a precise control of the applied training stimulus and, therefore, a more accurate control of the rodents' exercise intensity and duration. Nonetheless, using this method implies the animal removal from the habitual housing cage to a treadmill apparatus, whenever the exercise protocol is to be performed. This means that the animal will lose the possibility to perform wild-like activity patterns and, after the exercise onset, it will be restrained to the cage dimensions and will be forced to SB for most of the time. This type of training also relies on the use of adverse stimuli, such as electric shock to persuade running, implies training for extended periods of time in the absence of food and water, and typically happens during daytime hours when, usually, the animals are less active (Henriques-Coelho et al., 2008). Therefore, along with beneficial skeletal muscle functional adaptations (Moraska et al., 2000), the use of this forced-exercise model could lead to systemic alterations, like increased stress levels, replicating chronic stress situations and its deleterious-related effects, which affect neuroendocrine tissues and immune response (Moraska et al., 2000), particularly, increasing glucocorticoids production (Koh et al., 2005). An alternative to the forced exercise

is the voluntary running model. This technique allows animals to run voluntarily within their usual housing conditions while having access to food and water and, most importantly, do not use noxious stimuli to induce running. Indeed, despite the usual concern advocating that voluntary running, in cages equipped with the wheel system, is a stereotypic behaviour, expressing the animals' psychosis or obsession for being kept in captivity (Fisher et al., 2016), an interesting study demonstrated that wild animals often use the running wheels when placed in nature, mimicking the type of exercise bouts of the captive animals, and without extrinsic reward provided (Meijer & Robbers, 2014). Additionally, this model seems to enhance the animals' survival rate and decrease their body fat content (Narath et al., 2001). Nonetheless, it´s relevant to consider that the voluntary running results in an activity that consists of small, discontinuous bouts of running at velocities higher than those feasible with imposed, continuous treadmill running (Ferreira et al., 2012; Marques et al., 2011), and, contrary to forced exercise, do not guarantee an accurate control of the animals' physical activity intensity and duration. Collectively, despite the disadvantages of the wheel running system in precisely monitoring the exercise characteristics, this model is the one that better replicates the animals' wild type behaviour. Therefore, the mentioned data support our option to consider the animals of the control group those with access to the running wheel system, and the experimental group those without this device, contrary to most of the published literature (Abedi et al., 2004; Arsic et al., 2004; Koh et al., 2005). Moreover, the presented evidence, not only changes the idea that captive animals adopt a stereotypic conduct, advocating that voluntary running wheel is an optional behaviour, but also, considering that the modern common (sedentary) lifestyle and the lack of exercise are a major cause of disease worldwide, reinforces the use of this model in this field of research. The used model to induce SB did not allow to analyse if the effects induced on the SMR process were the result of the time spent in this condition previously to the injection, or instead, the consequence of low muscle recruitment after the CTX-induced injury. Nevertheless, since the main objective of our work was to mimic SB in general, and not to precisely study the muscle loading during the healing, we do not consider this fact a methodological limitation.

Regarding the injury inducing protocol, we opted for the local injections with venom from *Naja mossambica mossambica* snakes, also known as cardiotoxin, to produce skeletal muscle damage. This widely used procedure in the literature, effectively induces local skeletal muscle damage (Lepper et al., 2009; Sousa-Victor et al., 2014), through the generation of a vigorous and irreversible muscle contracture, possibly accomplished by major membrane-calcium imbalances (Lin Shiau et al., 1976). Besides this immediate and powerful toxic CTX-related effect to muscle fibres, it seems appealing to consider that the sustained muscle contraction, caused by the irreversible contracture, effectively contributes to an impaired blood flow (Wisnes & Kirkebo, 1976), exacerbating the physiopathology of venom-induced damage. In order to preserve the experimental procedure homogeneity, i.e., ensuring the injuries similarity, the infusion was induced by an appropriate syringe and needle, with the animals lightly anesthetised, in a subcutaneous muscle with the correspondent limb shaved, and all the injections performed by the same researcher. Nevertheless, considering the chance that the procedure homogeneity is very difficult to maintain – by the infusion of different amounts of CTX and/or different locations within the muscle tissue – the considered area for data collection was only that within clear muscle damage. Moreover, it seems important to acknowledge that the aim of our studies was not to compare the total damaged area produced by CTX infusion between groups, but to analyse the main features and characteristics of the healing process within those damaged regions.

Finally, regarding the used techniques for studying the SMR process, we selected a histological approach and not biochemical procedures. Histology techniques allow to accurately assess tissue organization, the existence of cellular damage and the type of infiltrating cells, allowing a more precise evaluation of these characteristics. Inversely, with biochemical techniques, and the inherent homogenised tissue, it would be impossible to precisely evaluate the cellular and molecular parameters studied.

Results Discussion

In general, the main outcomes of the presented studies support the hypothesis that SB negatively affects the SMR process in general, and, in particular, SB has adverse consequences on the myotubes formation rate, growth and maturation processes, the local cellular inflammatory response, and the fibrotic tissue accretion.

Our results demonstrate that the used protocol effectively induced marked skeletal muscle damage. The equivalent percentage of necrotic fibres, during the 1st day post-injection (dpi), indicated that the CTX-induced injury was similar in both groups, demonstrating that the experimental procedure was homogenous between groups, and that neither SB nor regular voluntary running changed the susceptibility to CTX toxicity. Curiously, current data evinces the protective effects of regular exercise and physical activity in both cardiac (Moreira-Goncalves et al., 2015; Powers et al., 2014) and skeletal muscle (Kavazis et al., 2014; Lawler et al., 2016). Our data did not demonstrate this protective effect, possibly because of the high dose of CTX infused to produce damage.

Regarding the inflammatory response, it was clear that SB exacerbated the Th1 phase, which was characterized by an increased number of both neutrophils and M1 macrophages compared to voluntary exercise control group. These results, considering the effects of regular exercise in increasing macrophages functions, like their phagocytic activity (Silveira et al., 2007; Sugiura et al., 2002; Woods et al., 2000), suggest that SB has evident adverse effects on the functionality of these immune cells, fact that was corroborated by the increased number of cells needed to accomplish the Th1 phase. Additionally, pro-inflammatory macrophages effectively increase both the tissue damage and the scar tissue formation (Wynn & Vannella, 2016). Consequently, considering these deleterious effects, the aggravated Th1 inflammatory response induced by SB may explain the postponement of the overall SMR in this group. Moreover, our data also demonstrated that regular exercise during SMR has significant effects on both neutrophils, M1 and M2 macrophages response, attenuating the Th1 phase –

diminishing the number of both neutrophils and M1 macrophages needed – and immediately intensifying the Th2 phase – promptly increasing the number of M2 macrophages. In agreement with our results, showing the quicker SMR on the control group, is the fact that anti-inflammatory macrophages are able to promote the tissue resolution phase during repair (Wynn & Vannella, 2016). Moreover, considering both the low grade chronic inflammation endorsed by SB (Booth et al., 2012) and the general anti-inflammatory effects of regular exercise (Gleeson et al., 2011), one could conceive the question whether these overall systemic alterations may be reflected locally on tissue response and, in the specific case of the skeletal muscle, on the immune inflammatory reaction during SMR. Interestingly, one example of this interaction may be explained by the modulatory effect of interleukin (IL)-10. Expression of IL-10, a potent anti-inflammatory cytokine – neutralising the production of cytokines and pro-inflammatory soluble mediators on neutrophils, monocytes and macrophages (Moore et al., 2001) – is effectively increased by exercise in both the skeletal muscle (Batista et al., 2010) and other tissues (Lira et al., 2009; Rojas-Ortega et al., 2015). Considering the presented evidences, it is difficult to justify whether this altered local inflammatory response in damaged skeletal muscle is the result of the systemic environment or the tissue milieu promoted by SB, being probably the results of both influences. Indeed, future studies should address this issue in order to clarify the importance of these SB-related detrimental effects.

It might be argued that the absence of SCs activity evaluation – usually identified by combined immunofluorescence of laminin and the paired domain transcription factor Pax7, respectively labelling the basal lamina and all quiescent and proliferating SCs (Yin et al., 2013) – could be a possible limiting factor of our work. Indeed, it is known that upon injury, SCs activate, proliferate and fuse to form functional regenerated myotubes (Persson, 2015), i.e., central nucleated fibres that, when stained with haematoxylin and eosin (H&E), display a predominantly basophilic (slightly purple) stain, possibly due to large amounts of RNA characteristic of actively differentiating and growing cells. Moreover, in addition to the SCs role in SMR, other myogenic stem cells, either resident or circulating, are known to contribute to the SMReg (Yin et al., 2013). Consequently, the lack

of SCs activity evaluation in our study only has limited the knowledge of the relative contribution of all these potential sources of myoblasts for myotubes formation, which, in fact, was not our objective. Therefore, as a mean to estimate overall myoblats activity, myotubes quantification was assessed by two different technics: (1) quantifying the central nucleated fibres in H&E-stained muscle sections, one of the histological hallmarks for evaluating regenerating muscle fibres (Grounds et al., 2008), and (2) counting myotubes expressing cluster differentiation (CD) 68 – commonly expressed in M1 macrophages, but also expressed in early developing cells, allowing them to attach to selectins during maturation (Gottfried et al., 2008) – by immunohistochemistry, which permitted the categorization of an earlier stage of myotubes development. The results obtained with this methodology demonstrated that SB impairs myotubes development and maturation processes. Indeed, when analysing the myotubes formation rate and their cross-sectional area in the experimental group, it seems plausible to assume that the reduced muscle recruitment delayed the myoblasts activity – as already demonstrated (Fujimaki et al., 2014) – which culminated with a decreased percentage of myotubes present in the 7^{th} dpi. Additionally, considering the CD-68 expression, our data showed that SB effectively delayed the myotubes maturation process, as confirmed by the decreased number of normal mature fibres within the original damaged area, on the 15^{th} dpi. A possible factor linking SB with the decreased myotubes formation is the lack of adequate mechanical tension. Indeed, the importance of mechanical tension in governing stem cells fate has already been addressed (Engler et al., 2006), indicating that these cells essentially need mechanical signs to improve their functionality. Moreover, other factor linking SB with the decreased myotubes muscle mass might be myostatin expression. This transforming growth factor-β family member, besides effectively reducing skeletal muscle growth (Lee & McPherron, 2001), is positively correlated with both age-related SCs dysfunction (McKay et al., 2012), and with impaired SMR (Li et al., 2008). Nonetheless, regular exercise, both endurance and resistance training, effectively reduces myostatin expression (Allen et al., 2011). Consequently, the probable increase in myostatin expression

caused by the SB-related reduced muscle recruitment seems to be hindering both the myoblasts activity and the muscle growth during the SMR.

Finally, our results also demonstrated that SB induced an excessive deposition of fibrotic tissue during SMR, and before considering the possible mechanisms explaining this occurrence, it seems relevant to acknowledge that the skeletal muscle organs, besides blood vessels and nerves, are also composed by connective tissue that forms the ECM. This important component is usually described as being composed by three layers: the epimysium, perimysium, endomysium, that are mostly composed by collagens (type I, III, IV, V, VI, XI, XII, XIV, XV and XVIII), proteoglycans, glycosaminoglycans, and collagen-proteoglycans interaction proteins (Gillies & Lieber, 2011). A narrow classification of the different ECM layers is difficult because, although each ECM layer seems to be composed by a predominant collagen type, there are collagens that have linking functions and, therefore, are dispersed between different layers. Type I and III collagens predominate in all ECM layers; the perimysium is composed mostly by type I collagen but type V collagen links it to the endomysium; the endomysium and the epimysium are composed mostly by type III collagen, and, finally, the basement membrane mostly constituted by type IV collagen and also by type VI, XV and XVIII (Gillies & Lieber, 2011). Finally, type VI collagen has been found dispersed throughout the endomysium and basement membrane of murine muscles (Urciuolo et al., 2013). Irrespective of this layer classification, several studies show that the ECM constituents are pivotal for the overall quality of skeletal muscle and, particularly, for the SCs activity. In fact, collagen VI integrity seems to be determinant for the skeletal muscles, considering that defects on its structure can cause muscle weakness and human myopathies (Grounds et al., 2005) and it absence can cause decreased muscle stiffness, reduced SCs self-renewal competence and impaired muscle regeneration (Urciuolo et al., 2013). Moreover, defects on collagen XV production may also impair the normal skeletal and cardiac muscle tissue development (Eklund et al., 2001). Additionally, resident fibroblasts effectively interact with SCs, increasing their proliferation, and positively contributing to an efficient and effective muscle regeneration (Murphy et al., 2011). Also, the proteoglycans molecules play a

critical role in the skeletal muscle fibrotic response, mainly associated with skeletal muscle dystrophies (Brandan & Gutierrez, 2013). Thus, regardless of their minor relative mass, the ECM seems to have a major influence on the different muscle cells functionality. Considering the described evidence, the lack of different collagen types evaluation is possibly the most relevant limitation of our study. Indeed, with the used picrosirius red staining methodology, all the collagens are identified, without any discrimination among the different types. Therefore, our results only show the overall changes in the amount of collagen content and do not allow to address any possible mechanisms relating the ECM characteristics with SMR. Notwithstanding, our results demonstrated that the collagen production was deeply increased by SB. Again, it is well known that the interactions between the ECM, the immune system, and the muscle SCs during the SMR, may profoundly affect the fibrosis accretion (Moyer & Wagner, 2011). Several causes and mechanisms of action may be linked to the obtained results. The mechanical tension imposed to fibroblast during contraction seems to be pivotal for nuclear signalling with gene expression and protein synthesis, which modulate the rates and type of collagen production, and the fibroblasts' cell cycle. Indeed, the increased muscle contraction modulates fibroblasts gene expression (Chiquet et al., 2009), and reduce the amount of fibrotic tissue accretion after injury (Richard-Bulteau et al., 2008). Accordingly, one can assume that reduced muscle use may compromise the fibroblasts functionality, unbalancing the SMR process towards the development of a more dysfunctional phenotype. Actually, this negative SB-related effect on the muscle phenotype, i.e., more fibrotic and with less muscle cross-sectional fibre area, as already been showed (Fonseca et al., 2012). Importantly, considering that the matrix metalloproteinases (MMPs) – proteases that mainly degrade collagen – functionality may have a dose-dependent relationship with exercise (Carmeli et al., 2005), it is plausible to assume that SB also blunts this mechanism of ECM remodelling. Myostatin expression may also be one of the factors linking the decreased skeletal muscle recruitment with the excessive fibrosis accretion. Indeed, myostatin expression is positively related with fibroblast proliferation and their production of muscle fibrosis (Li et al., 2008). Moreover, also supporting our results, is the fact that anti-

inflammatory macrophages are able to decrease fibrotic activity and increase the tissue resolution phase during repair (Wynn & Vannella, 2016). Despite the scarcity of studies aiming a thorough understanding of the interaction between different levels of physical activity or regular exercise on the ECM remodelling during SMR, collectively, our results showed that SB effectively affects fibroblast functionality, resulting in increased fibrosis throughout the time course of SMR, which apparently has an opposite relationship with SMReg

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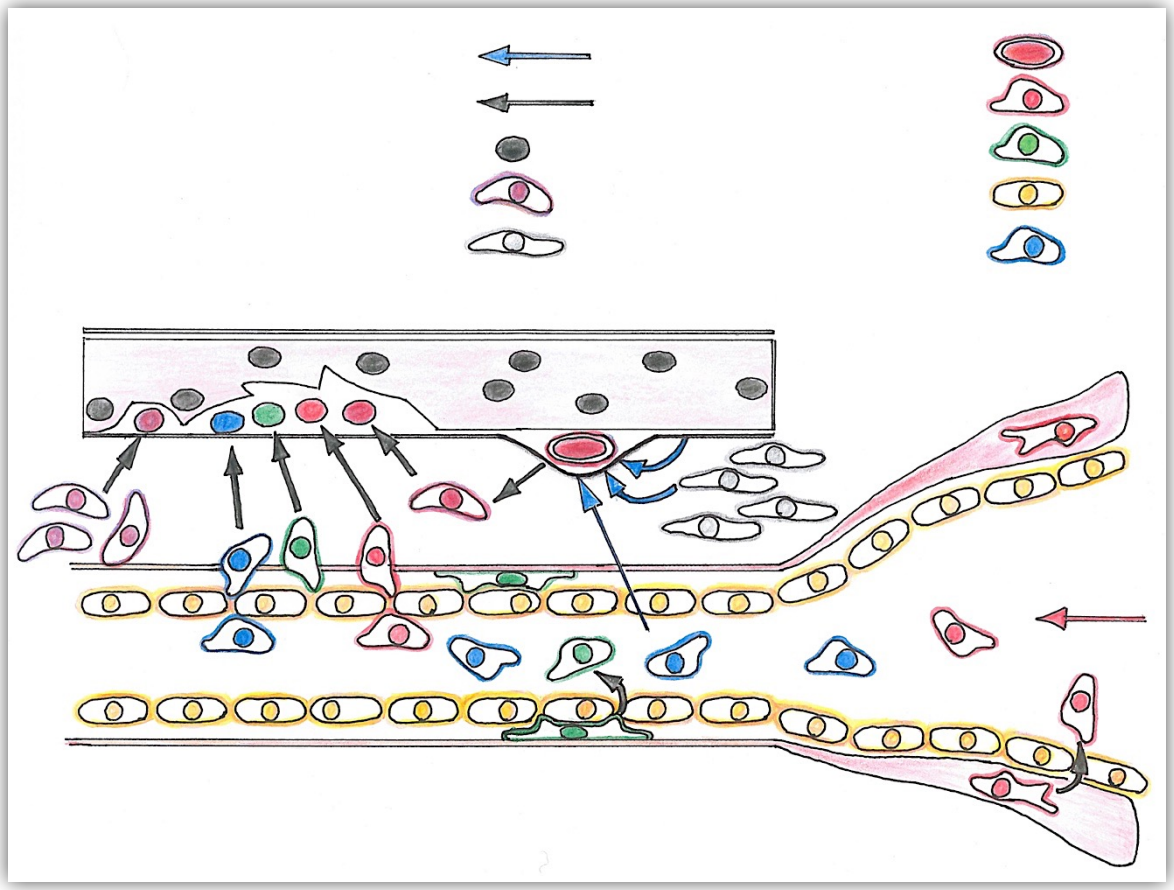
5. CONCLUSIONS AND FUTURE PERSPECTIVES



Conclusions

The main findings presented support that SB impairs rat SMR after CTX-induced damage, favouring fibrosis instead of SMReg. This global effect seems to be substantiated by the negative consequences of SB on: (1) the local inflammatory response: exacerbating the Th1 phase – through an augmented presence of neutrophils and M1 macrophages; and impairing the Th2 phase – blunting the macrophages polarization to the M2-biased phenotype; (2) the myotubes functionality: delaying their formation rate, maturation and growth; (3) the fibroblasts activity: intensifying the fibrotic tissue deposition. Bearing in mind the importance of skeletal muscles on the overall quality of life and ability to decrease the predisposition to diseases, our results clearly demonstrate that SB greatly prejudices their repair process, favouring scar tissue formation to the detriment of SMReg. Consequently, these SB-related outcomes during SMR will increase the changes of skeletal muscles to be progressively substituted by connective tissue when coping with daily wear and tear, culminating with the loss of their quality and maximal functionality throughout the lifespan.

Future perspectives

The effects of the different types of muscle loading in the SCs activity are well documented (Kadi et al., 2005), however, the mechanisms governing the effects of muscle loading/unloading in the overall cellular response during SMR are greatly unknown. Therefore, it would be relevant to address the mechanisms by which muscle function alters: (1) the inflammatory response – mainly the factors influencing the Th1 and Th2 phases, and, specifically, the macrophages polarization; (2) the fibroblasts activity – the aspects that influence their activity and the type of collagen produced upon SMR. It would also be appropriate to address if these SB-related effects on SMR are the result of the time spent in this condition prior to insult, or instead, the consequence of the low muscle recruitment during the healing process. Finally, it also seems important to study the dose-response relationship between muscle recruitment during the healing

process and proper SMR, in order to effectively answer the question: how much loading is necessary to effectively improve SMR?

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